

PATENT APPLICATION TRANSMITTAL LETTER

Docket Number: 4012-113 DIV1

I hereby certify that this paper is being deposited this date with the U.S. Postal Service as Express Mail in an envelope addressed to Assistant Commissioner for Patents, Box Patent Application, Washington, D.C., 20231 and Express mailed under the provisions of 37 CFR § 1.10

EL332463966US

Express Mail Label No.

Kit Stapleford

Name

Signature

October 29, 1999

Date

TO THE COMMISSIONER OF PATENTS AND TRADEMARKS:

Transmitted herewith for filing is the patent application of:

Applicant: Nnochiri Ekwuribe, et al.

Title: **"BLOOD-BRAIN BARRIER THERAPEUTICS"**Filing Date: **October 29, 1999 (Express Mail Filing Under 37 CFR § 1.10)**

This is a request for filing a Divisional Application under 37 C.F.R. § 1.60, of pending prior U.S. Patent Application No. 09/134,803, filed August 14, 1998 for "BLOOD-BRAIN BARRIER THERAPEUTICS" by Ekwuribe et al., as amended by a Preliminary Amendment dated July 27, 1999. Copies of the prior application and Preliminary Amendment are attached. These copies comprise a true and exact copy of the prior application as filed (specification, claims, drawings, declaration). The entire disclosure of the prior application, from which an oath or declaration is supplied, is considered a part of the disclosure of the accompanying divisional application and is hereby incorporated by reference. A verified statement establishing small entity status under 37 CFR § 1.9 and 37 CFR § 1.27 was submitted in the parent application and is still proper and desired.

Enclosed are:

- ☒ Specification, claims, and abstract.
☒ 10 sheet(s) of drawing(s).
☐ An Assignment of the invention to _____.
☒ A true and exact copy of the declaration and power of attorney from the parent case (U.S. Application No. 09/134,803)
☐ A certified copy of a _____ application.
☒ A verified statement to establish small entity status under 37 CFR § 1.9 and 37 CFR § 1.27.
☒ Other: copy of July 27, 1999 Preliminary Amendment in parent application

The following fee is calculated based on the cancellation of claims 23-72, as set forth in the attached preliminary amendment.

	Column 1	Column 2		Small Entity		Other Than Small Entity	
FOR:	NO. FILED	NO. EXTRA		RATE	FEE	RATE	FEE
BASIC FEE					\$380.00		\$790.00
TOTAL CLAIMS	24 - 20 =	4*		x \$9 =	36.00	x \$18 =	
INDEP. CLAIMS	4 - 03 =	1*		x \$39 =	39.00	x \$78 =	
<input type="checkbox"/> MULTIPLE DEPENDENT CLAIM PRESENTED				+ \$130 =	0	+ \$260 =	
				Total	\$455.00	Total	
					\$0.00		\$40.00
Fee for recording the assignment=					\$455.00		
TOTAL=							

* If the difference in Column 1 is less than zero, enter "0" in Column 2.

OR

OR

OR

OR

OR

PATENT APPLICATION TRANSMITTAL LETTER

- ☒ A check in the amount of \$455.00 (check number 6420) to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge any deficiency or credit any excess payment in connection with the filing of this application to Deposit Account No. 08-3284. A duplicate copy of this sheet is enclosed.

Bill Barrett

William A. Barrett, Reg. No. 42296

Date: Oct 29, 1999

INTELLECTUAL PROPERTY/TECHNOLOGY LAW

P.O. BOX 14329

Research Triangle Park, NC 27709

Telephone: (919) 419-9350

Facsimile: (919) 419-9354

Attorney File: 4012-113 DIV1

03/20/2000 10:24:00

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(c)) - - SMALL BUSINESS CONCERN

Docket No. 4012-113

Applicant:

Title:

Filing Date: August 14, 1998 (EXPRESS MAIL FILING UNDER 37 CFR 1.10)

I hereby declare that I am:



the owner of the small business concern identified below:

an official of the small business concern empowered to act on behalf of the concern identified below:

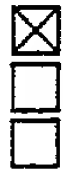
NAME OF SMALL BUSINESS CONCERN: Protein Delivery, Inc.

ADDRESS OF SMALL BUSINESS CONCERN: 2300 Englert Drive

Durham, North Carolina 27713

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in:



The specification filed herewith with title as listed above.

Application Serial No. _____ filed _____.

U.S. Patent No. _____ Issued _____.

If the rights held by the above-identified independent inventor are not exclusive, each individual, concern or organization having rights in the invention must file separate verified statements averring to their status as small entities, and that no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a non-profit organization under 37 CFR 1.9(e).

Each person, concern or organization having any rights in the invention is listed below:



No such person, concern, or organization exists.

Each such person, concern or organization is listed below:

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at any time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Christopher H Price

TITLE OF PERSON IF OTHER THAN OWNER CEO

ADDRESS OF PERSON SIGNING 2300 Englert Dr Durham, NC 27713

SIGNATURE [Signature] DATE 8.14.98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent Application of:)	
)	
Applicant:	Ekwuribe et al.)	
)	
Serial No.:	Not Assigned)	Examiner: Not Assigned
)	
Date Filed:	October 29, 1999)	Group Art Unit: Not Assigned
)	
Title:	Blood-Brain Barrier Therapeutics)	

EXPRESS MAIL CERTIFICATE

I hereby certify that I am mailing the attached documents to the Assistant Commissioner for Patents on the date specified below, in an envelope addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231, as Express Mailed under the provisions of 37 CFR 1.10.



Kit Stapleford



Date of Mailing

EL332463966US
Express Mail Certificate Number

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

Sir:

Preliminary to examination of this application, please amend the application as follows:

In the Claims

Cancel claims 23-63 and 66-72.

09429798-102999
666207-8645450

REMARKS

The applicants have cancelled claims 23-63 and 66-72, originally submitted in the application. Claims 1-22 and 64-65 are presently pending in the application.

As noted in the attached Transmittal Letter, the fee for filing the present application, including the fee for entry of the present claims, is \$455.00. A check (check no. 6420) for this amount is enclosed herewith. Please charge any deficiency in payment and credit any excess payment to Deposit Account No. 08-3284 of Intellectual Property/Technology Law.

The claims as presented are in form and condition for allowance. The Examiner is requested to contact the undersigned attorney at (919) 419-9350 to discuss the resolution of any issues which may arise incident to the formal allowance of this application, in order that this application may issue as quickly as possible.

Respectfully submitted,



William A. Barrett
Registration No. 42,296

**INTELLECTUAL PROPERTY/
TECHNOLOGY LAW**

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Phone: (919) 419-9350
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Attorney File: 4012-113 DIV1

04/26/2010 10:45:00 AM

In the Claims

Please amend the claims as follows:

5. (amended) The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is a peptide or protein [and the peptide is] selected from the group consisting of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminase, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystokinin, clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen activator, trypsin, vasopressin and analogues and active fragments thereof.
6. (amended) The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is an [Opioid] opioid receptor agonist, antagonist or partial agonist/partial antagonist.
10. (amended) The [The] amphiphilic drug-oligomer conjugate of claim 1 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a bond selected from the group consisting of: amide bond, carbamate bond, carbonate bond and ester bond.
11. (amended) The amphiphilic drug-oligomer conjugate of claim 1 wherein the oligomer moiety is coupled to the drug moiety by a bond selected from the group consisting of amide bond, carbamate bond, carbonate bond, and ester bond.

13. (amended) The amphiphilic drug-oligomer conjugate of claim 1 wherein the hydrophilic moiety is selected from the group consisting of sugars [or] and PEG₁₋₇.
14. (amended) The amphiphilic drug-oligomer conjugate of claim 1 wherein the hydrophilic moiety comprises a sugar and the sugar is selected from the group consisting of: amino sugars [and non-amino sugars].
15. (amended) The amphiphilic drug-oligomer conjugate of claim 1 wherein the oligomer is selected from the group consisting of:



wherein n=3 to 25 and m=1 to 6;



wherein n=3 to 25 and m=1 to 7;



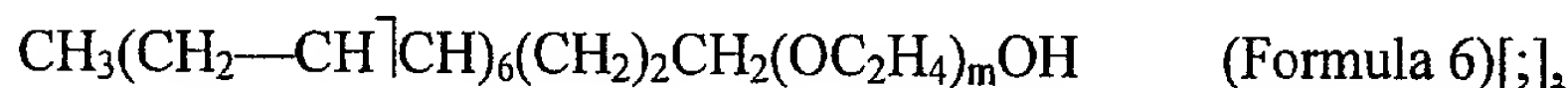
wherein n=3 to 25, m=1 to 7 and X=O or N;



wherein m=0 to 5 and R=cholesterol or adamantane; [or]



wherein m=0 to 5;



wherein m=0 to 7; and



wherein m=1 to 7 and X=N or O.

16. (amended) The [method] amphiphilic drug-oligomer conjugate of claim 1 wherein the hydrophilic moiety comprises a sugar and the sugar is selected from the group consisting of [amino sugars and] non-amino sugars.
17. (amended) The [method] amphiphilic drug-oligomer conjugate of claim 1 wherein the hydrophilic moiety comprises a monosaccharide.
18. (amended) [An] The amphiphilic drug-oligomer conjugate of claim 1 wherein the oligomer is selected from the group consisting of:



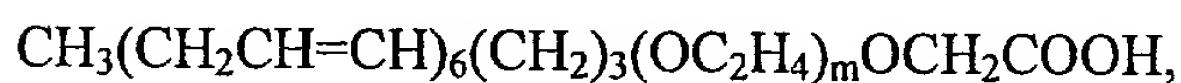
where m=1 to 7;



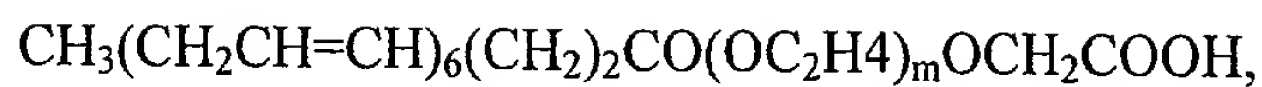
where m=1 to 7;



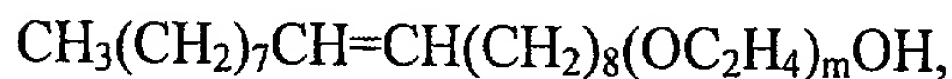
where m=1 to 6;



where m=1 to 6;



where m=1 to 6;



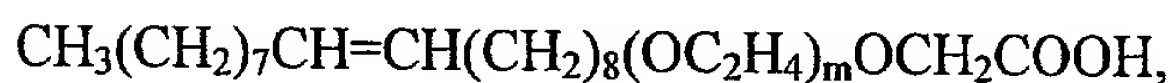
where m=1 to 7;



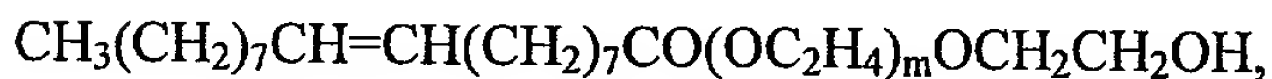
where m=1 to 7;



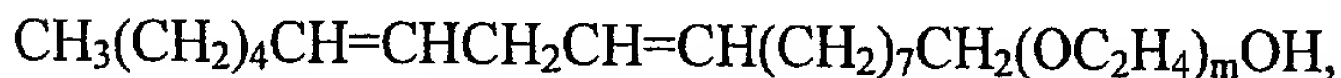
where m=1 to 6;



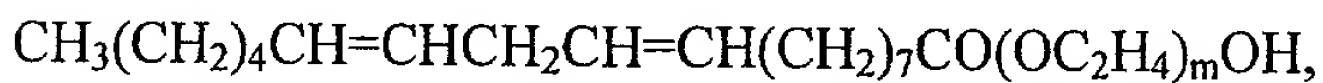
where m=1 to 6;



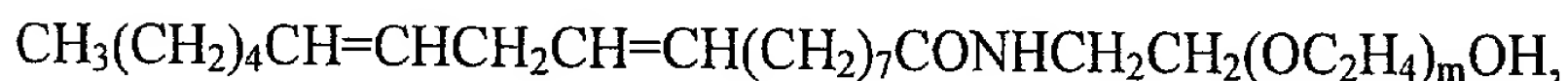
where m=1 to 6;



where m=1 to 6;



where m=1 to 7;



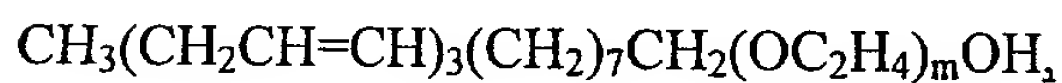
where m=1 to 6;



where m=1 to 6;



where m=1 to 6;



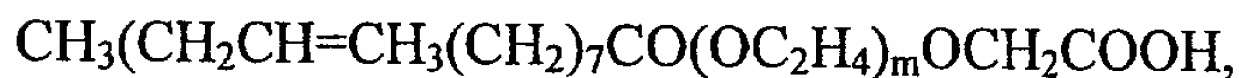
where m=1 to 7;



where m=1 to 7;



where m=1 to 6;

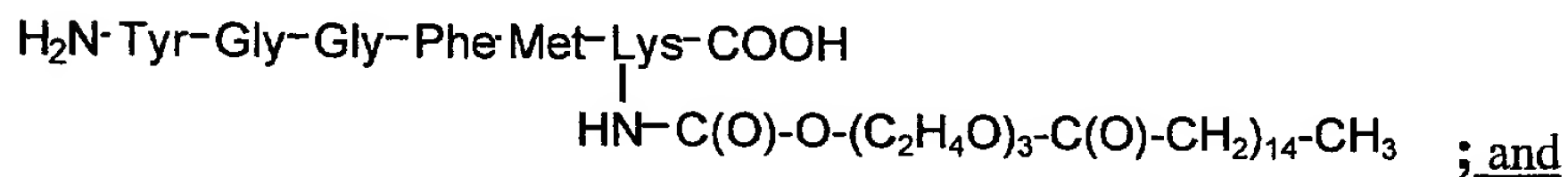
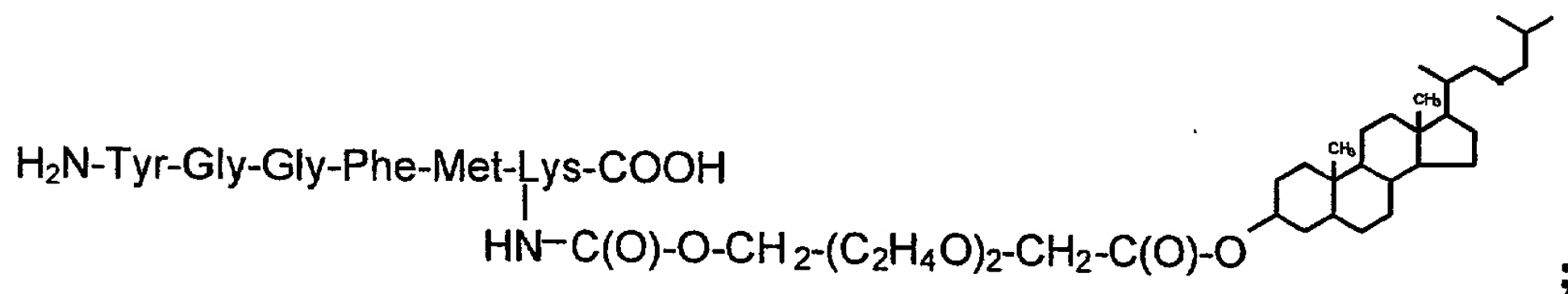
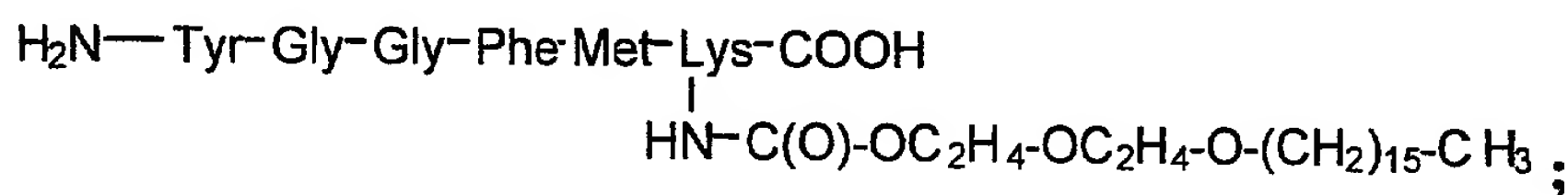
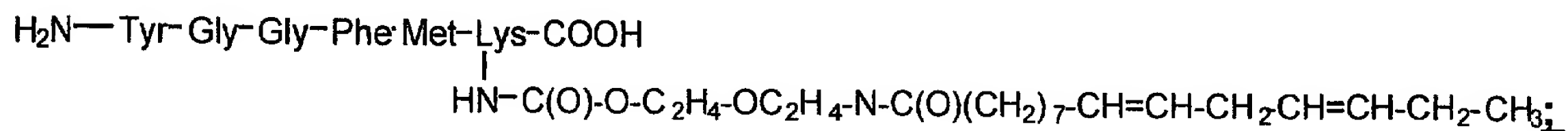
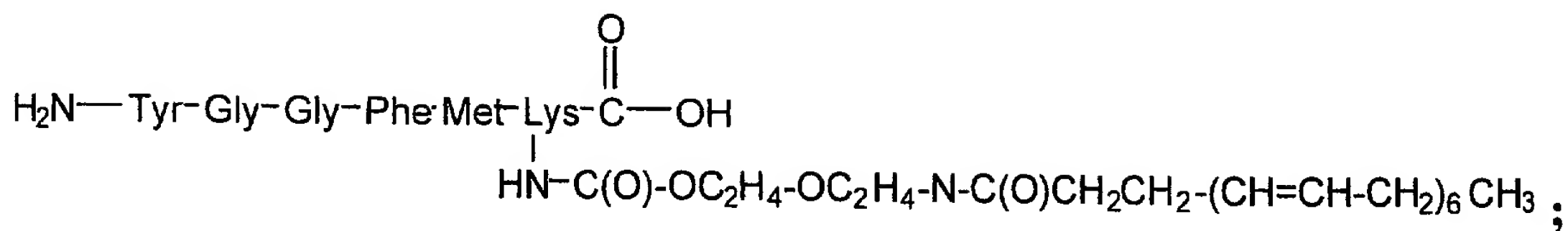


where m=1 to 6; and

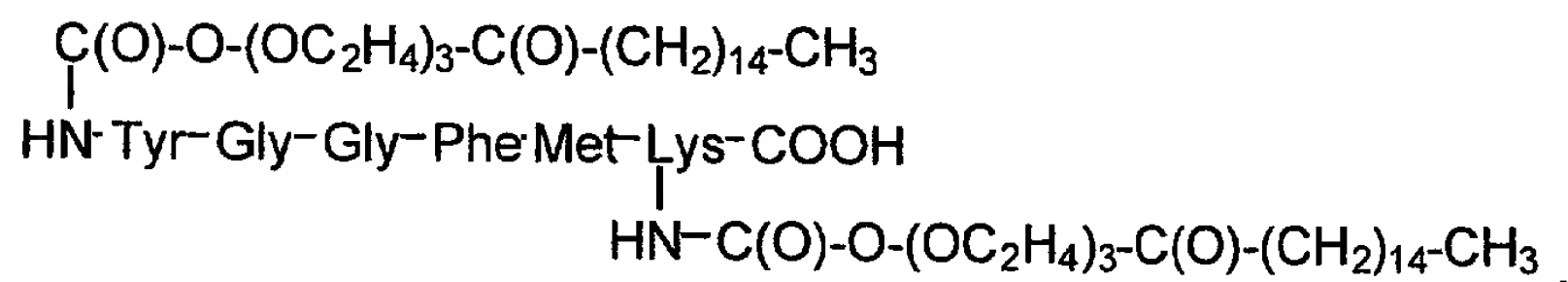


where m=1 to 6.

20. (amended) An amphiphilic oligomer-enkephalin conjugate selected from the group consisting of:



[; and]



24. (amended) The method of claim [18] 23 further characterized in that said conjugate exhibits activity in the without cleavage of the therapeutic compound from the oligomer.
25. (amended) The method of claim [18] 23 wherein the receptor is a G-protein coupled receptor.
26. (amended) The method of claim [18] 23 wherein the receptor is an [Opioid] opioid receptor.
27. (amended) The method of claim [18] 23 wherein the receptor is a [Opioid] opioid receptor; selected from the group consisting of δ , μ , and κ receptors.
28. (amended) The method of claim [18] 23 wherein the hydrophilic moiety is selected from the group consisting of sugar and PEG₁₋₇.
29. (amended) The method of claim [18] 23 wherein the hydrophilic moiety is selected from the group consisting of fatty acid, alkyl 1-26, cholesterol and adamantane.
30. (amended) The method of claim [18] 23 wherein the therapeutic compound is a peptide having an added N-terminal residue selected from the group consisting of proline[,] and alanine.
31. (amended) The method of claim [18] 23 wherein the therapeutic compound is a peptide or protein.
32. (amended) The method of claim [18] 23 wherein the therapeutic compound is a peptide [and the peptide is] or protein selected from the group consisting of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminease, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystokinin, clotting factors, dynorphins, endorphins,

endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen activator, trypsin, vasopressin, and analogues and active fragments of such peptides.

33. (amended) The method of claim [18] 23 wherein the amphiphilic oligomer is selected from the group consisting of:



wherein $n=3$ to 25 and $m=1$ to 6;



wherein $n=3$ to 25 and $m=1$ to 7;



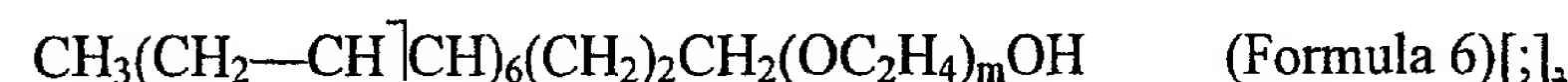
wherein $n=3$ to 25, $m=1$ to 7 and $X=\text{O}$ or N ;



wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 5;



wherein m=0 to 7; and



wherein m=1 to 7 and X=N or O.

34. (amended) The method of claim [18] 23 wherein the hydrophilic moiety is coupled to the hydrophobic moiety by a hydrolyzable bond.
35. (amended) The method of claim [18] 23 wherein the hydrophilic moiety is coupled to the hydrophobic moiety by a non-hydrolyzable bond
37. (amended) The method of claim [31] 36 wherein the therapeutic compound is a peptide or protein.
38. (amended) The method of claim [31] 36 wherein the therapeutic compound is a peptide [and the peptide is] or protein selected from the group consisting of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminease, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystokinin, clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen activator, trypsin, vasopressin, and analogues and active fragments of such peptides.
39. (amended) The method of claim [31] 36 wherein the therapeutic compound is [met⁵] enkephalin.
40. (amended) The method of claim [31] 36 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a hydrolyzable bond.

41. (amended) The method of claim [31] 36 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a non-hydrolyzable bond.
42. (amended) The method of claim [31] 36 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a bond selected from the group consisting of: amide bond, carbamate bond, carbonate bond and ester bond.
43. (amended) The method of claim [31] 36 wherein the oligomer moiety is coupled to the drug moiety by a bond selected from the group consisting of amide bond, carbonate bond, carbamate bond, and ester bond.
44. (amended) The method of claim [31] 36 wherein the lipophilic moiety is selected from the group consisting of fatty acids, C₁₋₂₆alkyls, and cholesterol.
45. (amended) The method of claim [31] 36 wherein the hydrophilic moiety is selected from the group [cobsisting] consisting of sugars, and PEG₁₋₇.
47. (amended) The method of claim [41] 46 wherein the therapeutic compound is [met⁵]enkephalin.
48. (amended) The method of claim [41] 46 wherein the lipophilic moiety is selected from the group consisting of fatty acids, C₁₋₂₆ alkyls, and cholesterol.
49. (amended) The method of claim [41] 46 wherein the one or more hydrophilic moieties are selected from the group consisting of sugars and PEG.
50. (amended) The method of claim [41] 46 wherein the hydrophilic moiety comprises a sugar and the sugar is selected from the group consisting of amino sugars and non-amino sugars.
51. (amended) The method of claim [41] 46 wherein the oligomer is selected from the group consisting of:



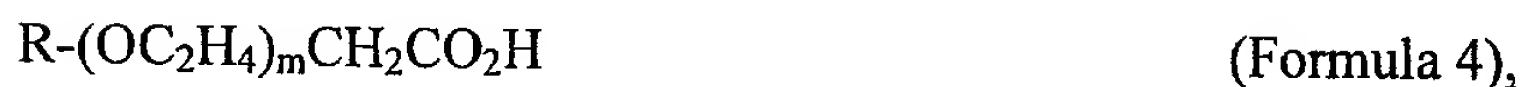
wherein $n=3$ to 25 and $m=1$ to 6;



wherein $n=3$ to 25 and $m=1$ to 7;



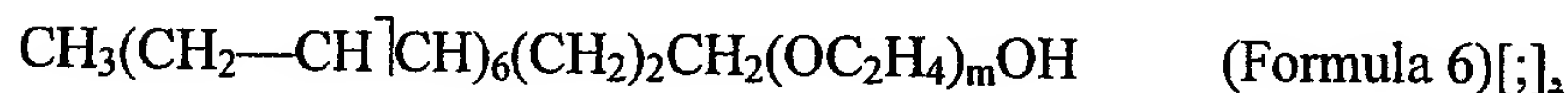
wherein $n=3$ to 25, $m=1$ to 7 and $X=\text{O}$ or N ;



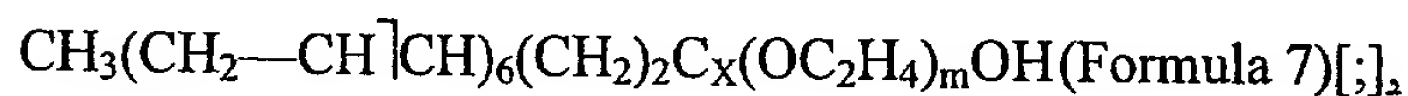
wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 4 and $\text{R}=\text{cholesterol}$ or adamantane ;

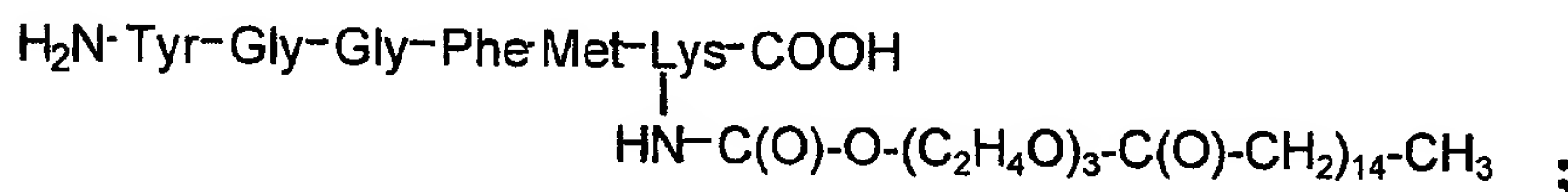
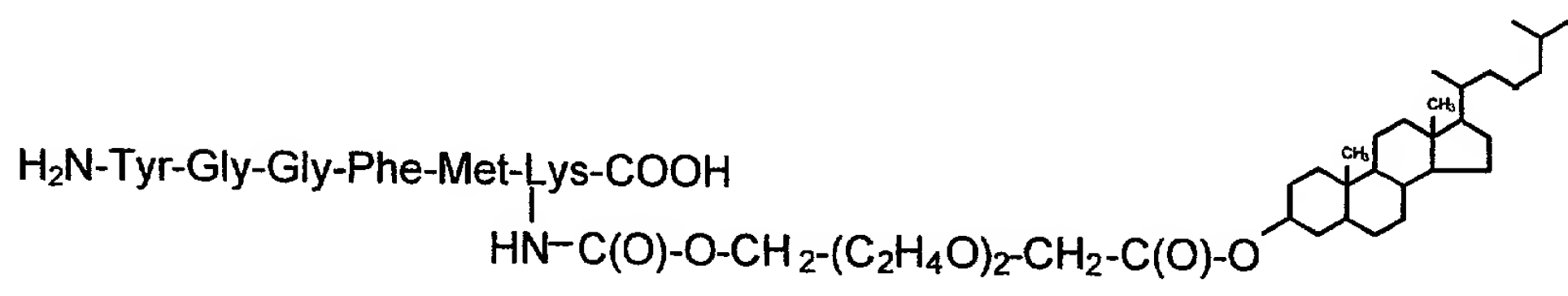
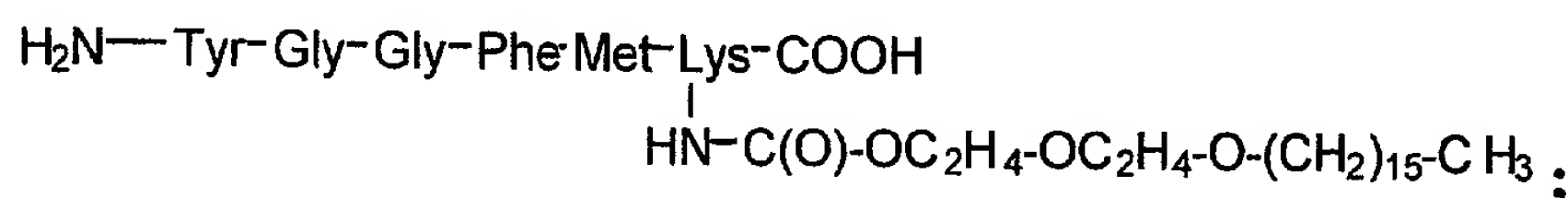
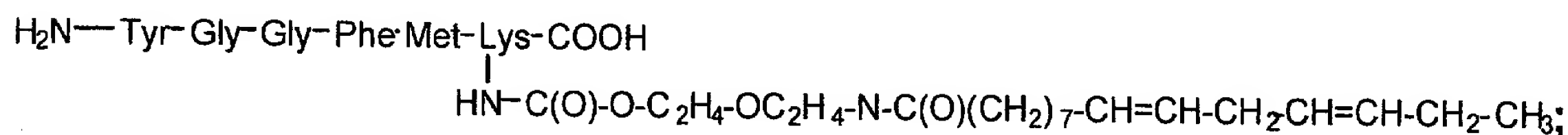
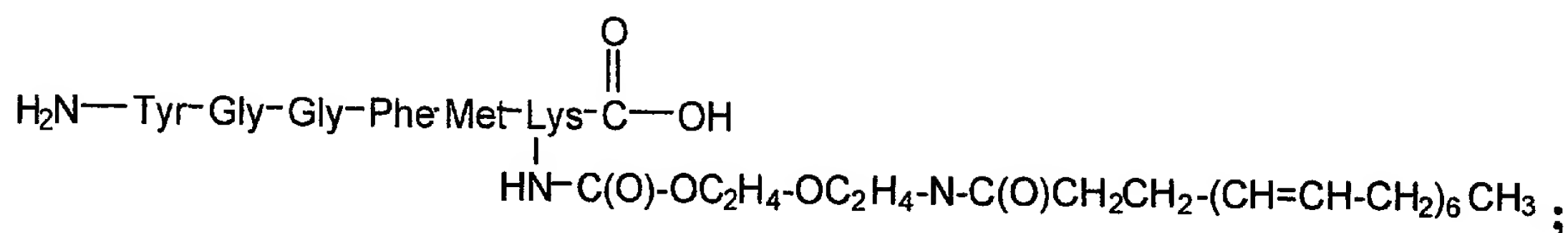


wherein $m=0$ to 7; and

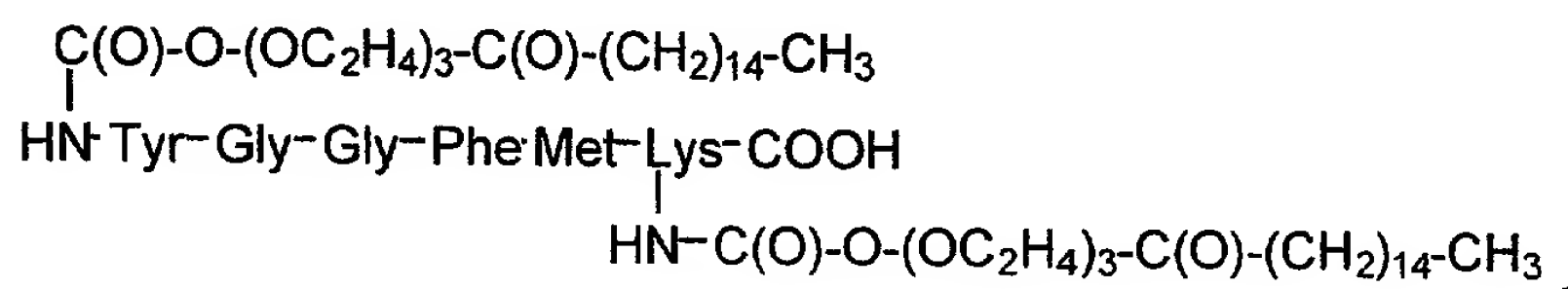


wherein $m=1$ to 7 and $X=\text{N}$ or O .

52. (amended) The method of claim 46 wherein the [An] amphiphilic oligomer-enkephalin conjugate is selected from the group consisting of:



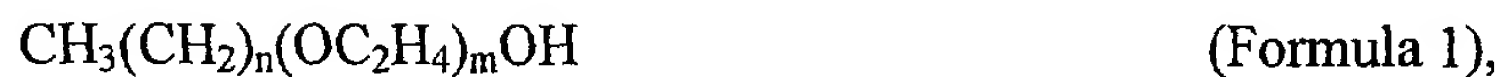
and



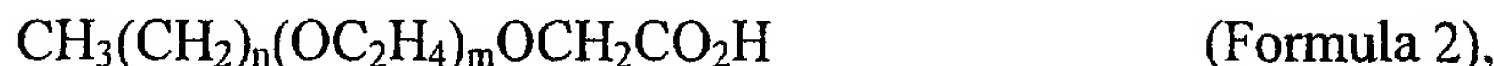
53. (amended) A method for altering the binding affinity of a peptide or protein to its receptor comprising conjugating the peptide to an amphiphilic oligomer comprising a lipophilic moiety coupled to a hydrophilic moiety.

54. (amended) The method according to claim 53 [48 further characterized in that] wherein the binding affinity is increased.
55. (amended) The method according to claim [48 further characterized in that] 53 wherein the binding affinity is reduced.
56. (amended) The method of claim [48] 53 wherein the [peptide is a] peptide or protein is an enkephalin.
57. (amended) The method of claim [48] 56 wherein the peptide or protein is selected from the group consisting of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminase, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystokinin, clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen activator, trypsin, vasopressin, and analogues and fragments of such peptides.
58. (amended) The method of claim [48] 56 wherein the [peptides] peptide is [met⁵]enkephalin.
59. (amended) The method of claim [48] 53 wherein the lipophilic moiety is selected from the group consisting of fatty acids, C₁₋₂₆alkyls, and cholesterol.
60. (amended) The method of claim [48] 53 wherein the hydrophilic moiety is selected from the group consisting of sugars or PEG₁₋₇.

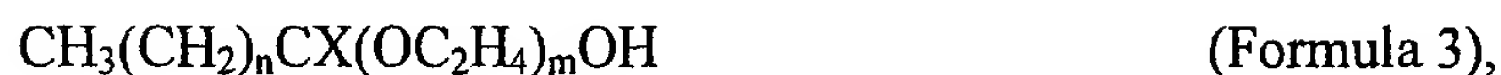
61. (amended) The method of claim [48] 53 wherein the oligomer is selected from the group consisting of:



wherein $n=3$ to 25 and $m=1$ to 6;



wherein $n=3$ to 25 and $m=1$ to 7;



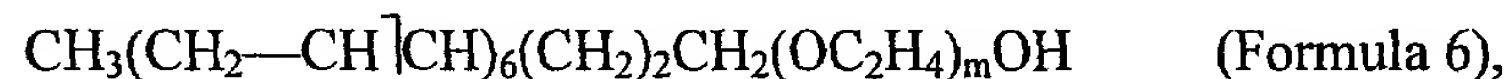
wherein $n=3$ to 25, $m=1$ to 7 and $X=\text{O}$ or N ;



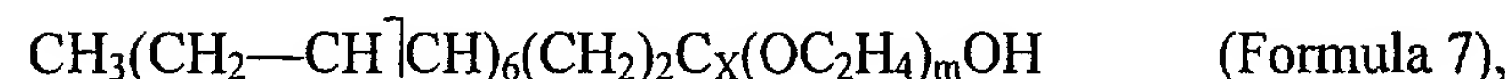
wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 5;



wherein $m=0$ to 7; and



wherein $m=1$ to 7 and $X=\text{N}$ or O .

62. (amended) The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is a peptide [and the peptide is] selected from the group consisting of:

Ac-Phe-Arg-Trp-Trp-Tyr-Lys—NH₂ ;

Ac-Arg-Trp-Ile-Gly-Trp- Lys —NH₂ ;

Trp-Trp-Pro-Lys-His-Xaa—NH₂ ,

wherein Xaa is a naturally-occurring amino acid;

Trp-Trp-Pro-Xaa—NH₂ ,

wherein Xaa is Lys or Arg;

Tyr-Pro-Phe-Gly-Phe-Xaa—NH₂ ,

wherein Xaa is a naturally-occurring amino acid;

(D)Ile-(D)Met-(D)Ser-(D)Trp-(D)Trp-Gly_n-Xaa—NH₂ ,

wherein n is 0 or 1 and wherein Xaa is Gly or the D-form-of a naturally-occurring amino acid;

(D)Ile-(D)Met-(D)Thr-(D)Trp-Gly-Xaa—NH₂ ,

wherein Xaa is Gly or the D-form of a naturally-occurring amino acid;

Tyr-A1-B2-C3—NH₂ ,

wherein A1 is (D)Nve or (D)Nle,

B2 is Gly, Phe, or Trp, and

C3 is Trp or Nap;

Pm and red {Me_xH_y-Tyr-(NMe)_z-Tyr-Xaa_z—NH₂}₂,

x is 0, 1, or 2,

y is 0, 1, or 2, and

z is 0 or 1, and

wherein Xaa is Phe, (D)Phe, or NHBzl, with the proviso that x and y together is never greater than 2; and

Trp-Trp-Pro-D4-His_z-Xaa_z—NH₂;

wherein z is 0 or 1,

wherein D4 is Lys or Arg, and

wherein Xaa is a naturally-occurring amino acid.

63. (amended) The method of claim 18 wherein the therapeutic compound is a peptide [and the peptide is] selected from the group consisting of:

Ac-Phe-Arg-Trp-Trp-Tyr-Lys—NH₂;

Ac-Arg-Trp-Ile-Gly-Trp- Lys —NH₂;

Trp-Trp-Pro-Lys-His-Xaa—NH₂,

wherein Xaa is a naturally-occurring amino acid;

Trp-Trp-Pro-Xaa—NH₂,

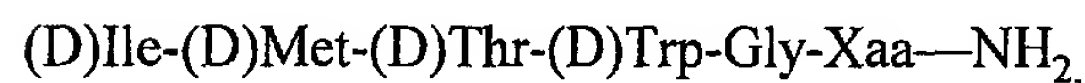
wherein Xaa is Lys or Arg;



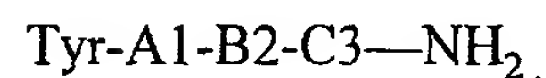
wherein Xaa is a naturally-occurring amino acid;



wherein n is 0 or 1 and wherein Xaa is Gly or the D-form-of a naturally-occurring amino acid;



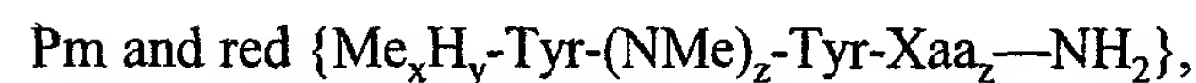
wherein Xaa is Gly or the D-form of a naturally-occurring amino acid;



wherein A1 is (D)Nve or (D)Nle,

B2 is Gly, Phe, or Trp, and

C3 is Trp or Nap;

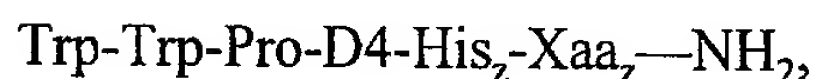


x is 0, 1, or 2,

y is 0, 1, or 2, and

z is 0 or 1, and

wherein Xaa is Phe, (D)Phe, or NHBzl, with the proviso that x and y together is never greater than 2;



wherein z is 0 or 1,

wherein D4 is Lys or Arg, and

wherein Xaa is a naturally-occurring amino acid.

Please add the following new claims 64 to 72:

64. The amphiphilic drug-oligomer conjugate of claim 1, wherein the therapeutic compound is an opioid.
65. The amphiphilic drug-oligomer conjugate of claim 1, wherein in the therapeutic compound is an enkephalin.
66. The method of claim 23 wherein the therapeutic compound is an opioid receptor agonist, antagonist or partial agonist/partial antagonist.
67. The method of claim 23 wherein the therapeutic compound is an enkephalin.
68. The method of claim 36 wherein the therapeutic compound is an opioid receptor agonist, antagonist or partial agonist/partial antagonist.
69. The method of claim 36 wherein the therapeutic compound is an enkephalin.
70. The method of claim 46 wherein the therapeutic compound is an opioid.

71. The method of claim 46 wherein the therapeutic compound is an enkephalin.

72. The method of claim 53 wherein the receptor is an opioid receptor.

REMARKS

The foregoing amendments to the claims are submitted to more precisely recite the applicant's claimed invention. The applicants note that in claims 39 and 58, the term "Met⁵" is in brackets, consistent with proper chemical nomenclature. It is not intended that the term in such brackets be deleted. The claim should be read "[Met⁵] enkephalin".

Claims 64-72 have been added herein to claim specific aspects of the invention. The amendments presented herein result in a total of nine (9) additional dependent claims, resulting in a fee of \$81.00. Accordingly, a check in the amount of \$81.00 (9 claims x \$9/claim) is submitted herewith. Please charge any deficiencies necessary for the entry of this amendment, or credit the excess, to Deposit Account No. 08-3284 of Intellectual Property/Technology Law.

It is requested that the examination of the application proceed on the basis of the application as amended by the above amendment.

Respectfully submitted,



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UNITED STATES PATENT APPLICATION

OF

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FOR

"BLOOD-BRAIN BARRIER THERAPEUTICS"

INTELLECTUAL PROPERTY / TECHNOLOGY LAW

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1. BACKGROUND OF THE INVENTION

5 1.1 FIELD OF THE INVENTION

The present invention relates to amphiphilic oligomer conjugates capable of traversing the blood-brain barrier ("BBB") and to methods of making and using such conjugates. The conjugates of the invention comprise therapeutic agents such as proteins, peptides, nucleosides, nucleotides, antiviral agents, antineoplastic agents, antibiotics, etc., and
10 prodrugs, precursors, derivatives and intermediates thereof, chemically coupled to amphiphilic oligomers.

1.2 DESCRIPTION OF THE RELATED ART

In the field of pharmaceutical and therapeutic invention and the treatment of disease states and enhancement of physiological conditions associated with the CNS, a wide
15 variety of therapeutic agents have been developed, including proteins, peptides, nucleosides, nucleotides, antiviral agents, antineoplastic agents, antibiotics, etc., and prodrugs, precursors, derivatives and intermediates thereof.

Additionally, the many known neuroactive peptides offer additional possibilities for useful therapeutic agents. Such neuroactive peptides play important biochemical roles in the
20 CNS, for example as neurotransmitters and/or neuromodulators. Delivery of this diverse array of peptides to the CNS provides many opportunities for therapeutic benefit. For example, delivery of endogenous and synthetic opioid peptides, such as the enkephalins, can be used to effect analgesia.

However, a number of obstacles currently limit the use of many compounds for use as
25 CNS therapeutic agents.

First, the brain is equipped with a barrier system. The brain barrier system has two major components: the choroid plexus and the blood-brain barrier (BBB). The choroid

plexus separates cerebrospinal fluid (CSF) from blood and the BBB separates brain ISF from blood.

The BBB has about 1000 times more surface area than the choroid plexus and is the primary obstacle to delivery of therapeutic compounds to the CNS. The BBB acts as a selective partition, regulating the exchange of substances, including peptides, between the CNS and the peripheral circulation. The primary structure of the BBB is the brain capillary endothelial wall. The tight junctions of brain capillary endothelial cells prevent circulating compounds from reaching the brain ISF by the paracellular route. Furthermore, recent work suggests the existence of a physiological barrier at the level of the basal lamina, in addition to the barrier provided by the tight junctions. Kroll *et al.*, *Neurosurgery*, Vol. 42, No. 5, p.1083 (May 1998). Other unique characteristics of the BBB include lack of intracellular fenestrations and pinocytic vesicles and a net negative charge on the luminal surface of the endothelium. *Id.*

The mechanisms by which substances may traverse the BBB may generally be divided into active and passive transport mechanisms. Lipophilic molecules readily traverse the BBB by passive transport or diffusion through the endothelial plasma membranes. In contrast, hydrophilic molecules, such as peptides, typically require an active transport system to enable them to cross the BBB. Certain larger peptides, such as insulin, have receptors on the luminal surface of the brain capillaries which act as active transcytosis systems.

Diffusion of many therapeutic compounds, such as peptides, across the BBB is also inhibited by size. For example, cyclosporin, which has a molecular weight of ~1200 Daltons (Da), is transported through the BBB at a much lower rate than its lipid solubility would predict. Such divergence between lipid solubility and BBB permeation rates is probably due to steric hinderances and is common where the molecular weight of a compound exceeds 800-1000 Da.

A further barrier to peptide delivery to the CNS is metabolic instability. In particular, before peptides injected into the blood reach the CNS, they must survive contact with enzyme degrading enzymes in the blood and in the brain capillary endothelium. BBB enzymes are known to degrade most naturally occurring neuropeptides. Orally

administered peptides face additional barriers discussed below. Metabolically stabilized peptides may exhibit increased resistance to certain enzymes; however, it has not been possible to protect peptides from the wide range of peptide-degrading enzymes present in the blood and BBB.

- 5 Another difficulty inherent in delivering peptides to the BBB is that successful transcytosis is a complex process which requires binding at the luminal or blood side of the brain capillary endothelium, movement through the endothelial cytoplasm, and exocytosis at the abluminal or brain side of the BBB. Peptides may bind to the luminal membrane of the brain capillary endothelium or undergo binding and endocytosis into
10 the intracellular endothelial compartment without being transported into the CNS.

In any event, many currently existing drug substances, especially peptides, are unable to overcome these structural and metabolic barriers to enter the BBB in sufficient quantities to be efficacious. There is therefore a need for pharmaceutical compositions which can
15 (1) withstand degradative enzymes in the blood stream and in the BBB and (2) which can penetrate through the BBB in sufficient amounts and at sufficient rates to be efficacious.

Many attempts have been made in the art to deliver therapeutic compounds, such as peptides, to the CNS with varying levels of success. Such attempts can generally be grouped into two categories: invasive and pharmacological.

- 20 Invasive delivery strategies include, for example, mechanical procedures, such as implantation of an intraventricular catheter, followed by pharmaceutical infusion into the ventricular compartment. Aside from general considerations relating to the invasiveness of mechanical procedures, a major difficulty with mechanical approaches is the lack of peptide distribution. For example, injection of peptides into the CSF compartment
25 results in very little distribution beyond the surface of the brain. This lack of distribution is due in part to rapid exportation of peptides to the peripheral circulation.

Another invasive strategy for delivering therapeutic compounds to the CNS is by intracardoid infusion of highly concentrated osmotically active substances, such as mannitol or arabinose. Their high local concentration causes shrinkages of the brain

capillary endothelial cells, resulting in a transient opening of the tight junctions which enable molecules to traverse the BBB. Such procedures have considerable toxic effects, including inflammation, encephalitis, etc. Furthermore, such procedures are not selective: the opening of the tight junctions of the BBB permits many undesirable substances to cross the BBB along with the therapeutically beneficial molecule. For a recent review of osmotic opening and other invasive means for traversing the BBB, see Kroll, Robert A. *Neurosurgery*, Vol. 42, No. 5, May 1998.

While the risks involved in these invasive procedures may be justified for life-threatening conditions, they are generally not acceptable for less dramatic illnesses. There is therefore a need for less invasive, non-mechanical and safer means for enabling therapeutic compounds to cross the BBB.

As noted above, lipophilic substances can generally diffuse freely across the BBB. Accordingly, a common pharmacological strategy for enabling peptides to traverse the BBB is to chemically modify the peptide of interest to make it lipid-soluble. Hydrophilic drug substances have been derivatized with short chain or long chain fatty acids to form prodrugs with increased lipophilicity.

Prodrugs are biologically inert molecules which require one or more metabolic steps to convert them into an active form. A difficulty with the prodrug approach to crossing the BBB is that the cleavage necessary to yield an active drug may not occur with sufficient efficiency and accuracy to produce an efficacious amount of the drug.

There is therefore a need for modified stable therapeutic compounds, such as peptides, which are capable of traversing the BBB but which retain all or part of their efficacy without requiring metabolic steps to convert them into an active form.

A further difficulty with lipidized prodrugs is that they pass in and out of the CNS so readily that they may never reach sufficient concentration in the CNS to achieve their intended function. For example, previous attempts have been made to engineer enkephalin conjugates which can traverse the BBB. See Partridge, W.M., "Blood-Brain Barrier Transport and Peptide Delivery to the Brain," *Peptide-Based Drug Design: Controlling Transport and Metabolism*, p. 277 (1995). However, these strategies

required the subcutaneous delivery of frequent and massive doses of peptide to induce analgesia. Frequent and/or massive dosing is inconvenient to the patient and may result in serious side effects.

5 There is therefore a need in the art for means for enabling therapeutic agents, such as peptides, to cross the BBB in a controlled manner which permits accumulation of sufficient quantities of the therapeutic in the brain to induce the desired therapeutic effect.

10 Another pharmacological method for delivering peptides across the BBB is to covalently couple the peptide of interest to a peptide for which a specific receptor-mediated transcytosis system exists. For example, it is theoretically possible to attach β -endorphin, which is not normally transported through the BBB, to insulin to be transported across the BBB by insulin receptor-mediated transcytosis. Upon entry into the brain interstitial space, the active peptide (β -endorphin) is then released from the transport vector (insulin) to interact with its own receptor.

15 However, the difficulty with this system is designing a chimeric molecule which can become detached upon entry into the interstitial space; to the inventor's knowledge, this has not yet been achieved. Additionally, the poor stoichiometry of the neuropeptide to the carrier molecule limits the mass of the target peptide. Furthermore, receptor-mediated cellular transport systems typically have physiologically limited transport capacity. This is a rate-limiting factor which can prevent entry of pharmaceutically active amounts of peptide.

20 There is therefore a need in the art for means for enabling therapeutic substances, such as peptides, to cross the BBB by diffusion so as to avoid the limitations inherent in receptor-mediated transport.

25 Other pharmacological strategies include using an active fragment of a native peptide; modification of a native peptide to increase blood-brain barrier (BBB) transport activity; and delivery of a gene encoding the neuropeptide to the brain.

Oral administration is a desirable and convenient route of administration; however, orally delivered peptides must overcome a series of barriers before they can enter the blood stream. Such peptides must survive proteolysis and the acidic environment of the stomach, gastric and pancreatic enzymes, exo- and endopeptidases in the intestinal brush border membrane.

There is therefore a need for orally administered peptides which can also resist proteolytic enzymes in the blood and BBB and which can traverse the BBB in sufficient quantities to provide broad distribution of drugs into the entire brain parenchyma.

Methionine-enkephalin and leucine-enkephalin are naturally occurring analgesic pentapeptides. These peptides and their analogs are known to act as neurotransmitters or modulators in pain transmission. Their analgesic properties are short in duration. When administered by intracerebroventricular injection, the duration of their action is also transient.

These properties make the enkephalins attractive compounds for use as therapeutic agents, for mediating analgesia and providing a viable alternative to morphine. However, in order to deliver enkephalins across the BBB, they must be protected against rapid degradation by aminopeptidases and enkephalinases. Furthermore, since enkephalins are hydrophilic peptides, they must be modified to provide them with increased lipophilic characteristics before they can passively diffuse across the BBB into the CNS.

The attractive therapeutic properties of enkephalins have been known for some time, and many investigators have attempted to enhance the ability of enkephalins to traverse the BBB.

Schroder et al., *Proc. Int. Symp. Control Rel. Biact. Material*, Vol. 23, p. 611 (1996) teaches that Dalargin, a leu-enkephalin analogue can be incorporated in nanoparticles formed by polymerization of butylanoacrylate. The particles are coated with polysorbate, a penetration enhancer. Analgesic activity is obtained after intravenous administration. Unlike the present invention, however, the Schroder peptide must be chemically bound

to the polymeric material or to the polysorbate. The formulation is therefore a physical mixture of active drug and polymeric material.

5 Tsuzuki *et al.*, *Biochem. Pharm.* Vol. 41, p. R5 (1991) teaches that analogues of leu-enkephalin can be derivatized with adamantane moiety to obtain lipophilic enkephalin that shows an antinociceptive effect after subcutaneous administration. Modification at the N-terminus abolishes activity while the derivative at the C-terminus through ester bond retains activity. It is postulated that the activity is obtained after cleavage of the adamantane moiety. The derivative is therefore a prodrug, a concept not consistent with aspects of the present invention in which the therapeutic conjugate retains the activity of
10 the native peptide.

Prokai-Tatra, *J. M. Chem*, Vol. 39, p. 4777 (1996) teaches that a leucine-enkephalin analogue can be modified with chemical delivery system which is based on a retrometabolic drug design. The enkephalin analogue is derivatized with a dihydropyridine moiety at the N-terminus and a lipophilic moiety at the C-terminus. After
15 intravenous administration of the conjugate, analgesic response is observed. It is postulated that the lipophilic modification at the C-terminus enables penetration into the CNS, while the dihydropyridine moiety undergoes oxidative transformation to generate a charged moiety which restricts the peptides from effluxing into the circulatory system. Cleavage of the peptide from this moiety restores the observed analgesic activity. The
20 derivatized peptide is inactive and regains activity only after metabolic transformation. The product is therefore a pure prodrug, requiring metabolic transformation to transform it into an active form.

U.S. Patent 4,933,324 to Shashoua teaches that certain natural fatty acids can be conjugated to neuroactive drugs. A highly unsaturated fatty acid of twenty-two (22)
25 carbon chain length is particularly preferred. Administration of the conjugate shows absorption into the brain. As is the case with adamantane conjugation, this approach requires metabolic transformation of the prodrug conjugate of enkephalin to restore the activity of the enkephalin peptide.

There is therefore a compelling need in the art for pharmaceutically acceptable and effective therapeutic/diagnostic compositions capable of traversing the BBB without substantial loss or diminution of their therapeutic or diagnostic character.

2. SUMMARY OF THE INVENTION

5 The present invention broadly relates to therapeutic and/or diagnostic drug-oligomer conjugates wherein a drug molecule is covalently bonded to an oligomer to form an amphiphilic conjugate. In one aspect, the oligomer comprises at least one lipophilic moiety and at least one hydrophilic moiety, and the size and nature of the amphiphilic and lipophilic moieties is so selected as to impart an amphiphilic nature to the resulting
10 conjugate.

The present invention relates generally to amphiphilic drug-oligomer conjugates capable of traversing the BBB and to methods of making and using such conjugates.

In one aspect, the therapeutics are neuroactive drugs, proteins, peptides and especially enkephalin analogues. The conjugates are stable in the environment of the bloodstream
15 and resist degradation by the enzymes of the BBB and in the CNS. Furthermore, the conjugates readily traverse the BBB.

In one aspect, the drug-oligomer conjugates produce their intended pharmacological effect without undergoing metabolic cleavage of the oligomer.

In another aspect, the lipophile and hydrophile are connected by a labile, hydrolyzable
20 bond. When the bond is hydrolyzed in the CNS, the hydrophile remains attached to the drug.

The amphiphilic oligomers are composed of lipophilic and hydrophilic moieties. The lipophilic moieties are preferably natural fatty acids or alkyl chains. Preferably, the fatty-acid moiety is a straight chain molecule having (saturated or unsaturated) carbon atoms
25 and suitably ranges from four (4) to twenty-six (26) carbon atoms. Most preferably, the fatty acid has from fourteen (14) to twenty-two (22) carbon atoms.

The hydrophilic moieties are preferably small segment of polyethylene glycol (PEG), preferably having 1-7 PEG units, and more preferably 1-5 PEG units. The length and composition of the lipophilic moieties and the hydrophilic moieties may be adjusted to obtain desired amphiphilicity.

- 5 In another aspect, a cholesterol or adamantane moiety is substituted for straight chain fatty acid portion of the oligomers.

Examples of preferred oligomers are as follows:



wherein $n=3$ to 25 and $m=1$ to 7;



wherein $n=3$ to 25 and $m=1$ to 6;



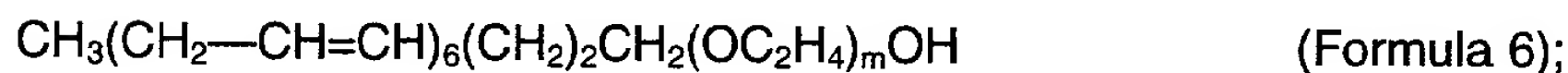
wherein $n=3$ to 25, $m=1$ to 7 and $\text{X}=\text{O}$;



15 wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 5;



wherein $m=0$ to 7;

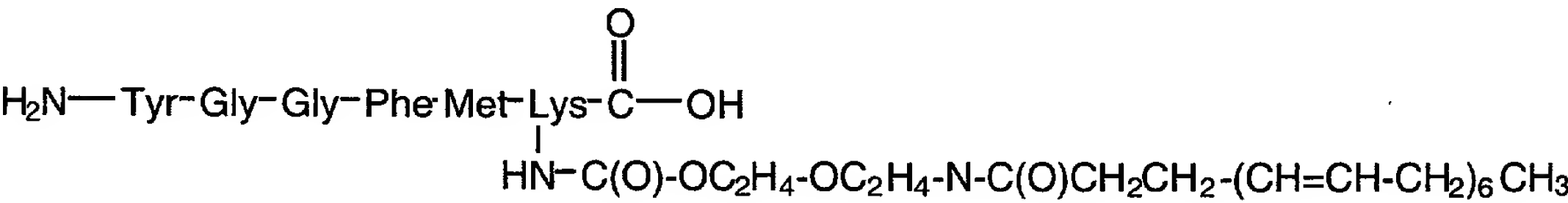


wherein m=1 to 7 and X=N or O.

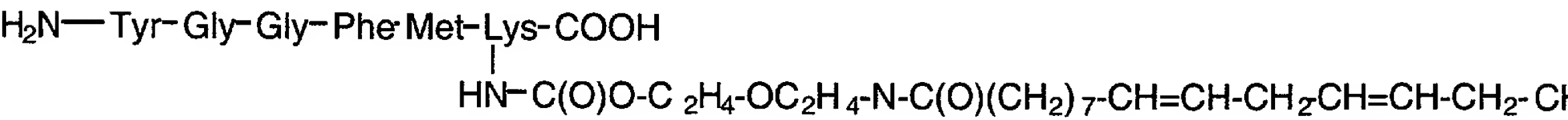
Other unsaturated fatty acid moieties which can be used according to the present invention include oleic, linoleic, and linolenic.

For example, in one aspect, the lipophile and hydrophile are connected by hydrolyzable bonds. It is preferred to provide hydrolyzable bonds between the fatty acid and hydrophilic moieties. This permits hydrolysis to occur after penetration into the CNS, thus releasing the active peptides with the hydrophilic group still attached to the peptide. As a result, the peptide acquires a more hydrophilic character and efflux to the circulatory system is thereby hindered.

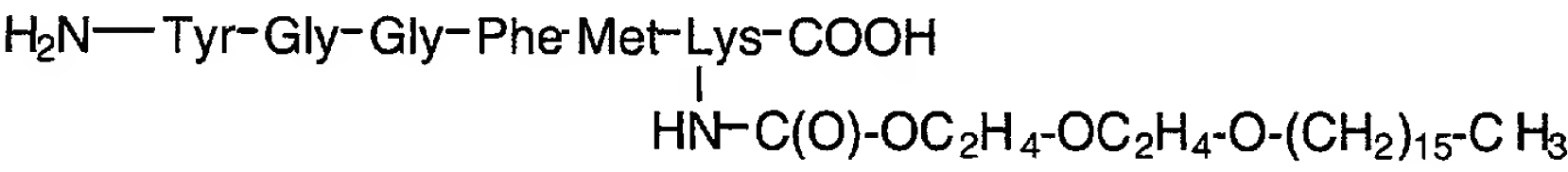
Exemplary conjugates having non-hydrolyzable bonds are as follows:



DHA MET-ENKEPHALIN-LYS



LINOLEIC MET-ENKEPHALIN-LYS

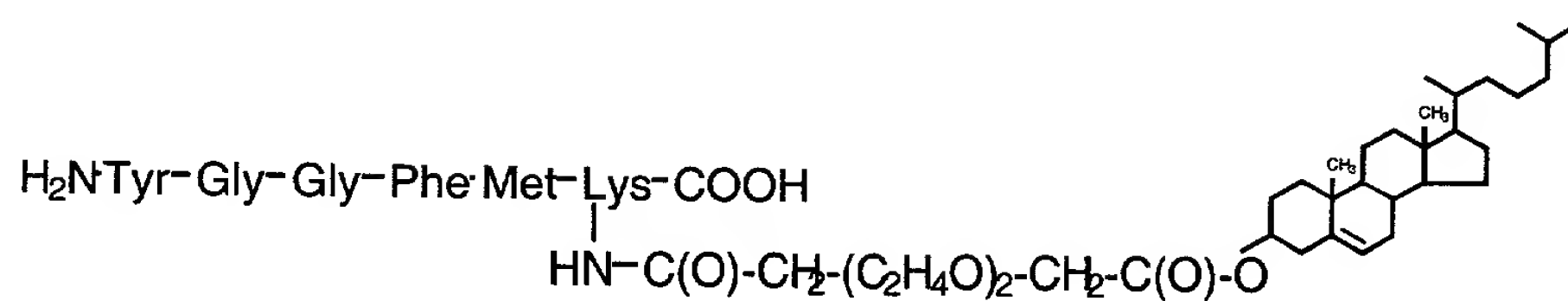


CETYL MET-ENKEPHALIN-LYS

5

In another aspect, the lipophile and hydrophile are connected by hydrolizable bonds.
For example:

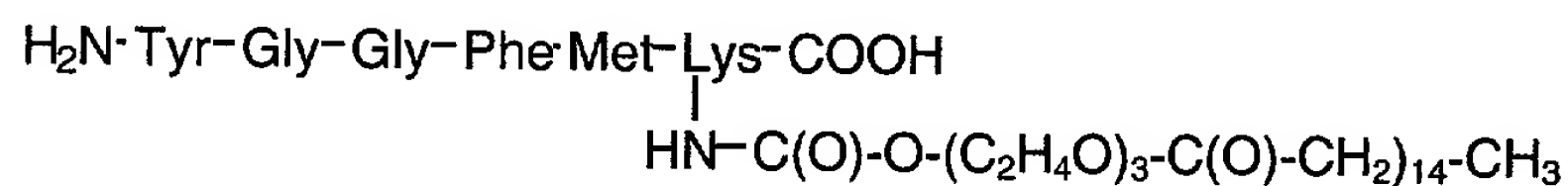
10



15

CHOLESTEROL MET-ENKEPHALIN-LYS

20



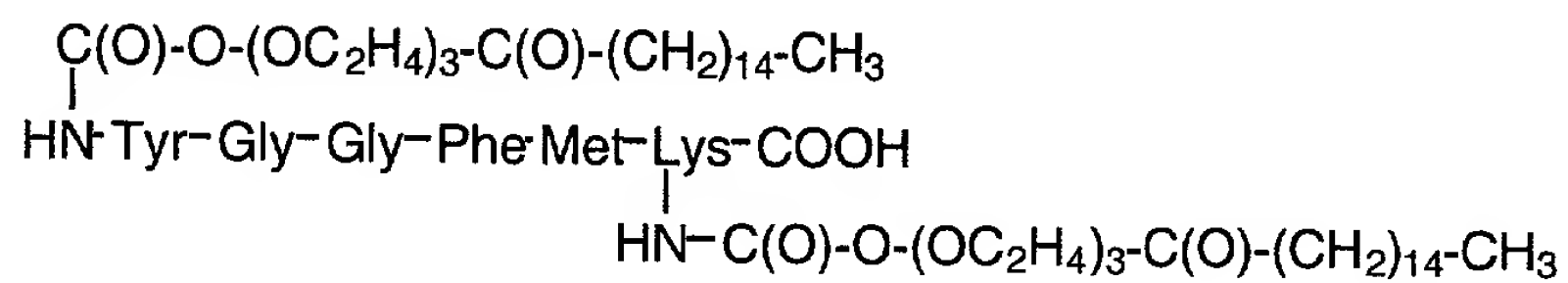
25

PALMITATE-TEG MET-ENKEPHALIN-LYS

30

35

40



DI-PALMITATE-TEG MET-ENKEPHALIN-LYS

In one aspect, the covalent bond between the oligomer and the drug is preferably amide (a carboxy group of the oligomer is linked to an amine group of the peptide drug), or carbamate (an chloroformate group of the oligomer is linked to an amine group of the peptide drug). In general, the derivitizable amine group of the peptide is the amine of the N-terminus or a nucleophilic amino residue, usually found on the epsilon amino residue of a lysine residue.

In another aspect, an ester (a carboxy group of the peptide is covalently coupled to a hydroxyl group of the oligomer or a carboxy group of the oligomer is covalently coupled to a hydroxyl group of the drug), amide (a carboxy group of the oligomer is linked to an amine group of the drug) or carbamate (an chloroformate group of the oligomer is linked to an amine group of the drug) bond is provided for non-peptide drugs.

For the enkephalin analogues, the preferred peptides are leu-enkephalin lysine and met-enkephalin lysine. The amino side chain of the lysine is preferably utilized in bonding.

The amphiphilic drug-oligomer conjugate may comprise multiple oligomers of differing compositions.

In another aspect, the amphiphilic drug-oligomer conjugates moieties are configured as follows:



Wherein R=alkyl₁₋₂₆, cholesterol or amantane.

In another aspect of the amphiphilic oligomer moieties are sugar moieties coupled to natural fatty acids and segments of polyethylene glycol. The PEG moiety serves to

increase the amphiphilicity of the fatty sugar. Examples of arrangements including sugar moieties are provided in Figures 1A-1C.

In another aspect, PEG is used as a spacer group in the amphiphilic conjugate and the length and number of the PEG moieties can be varied to refine the amphiphilicity of the conjugate. Increasing the number of PEGs increases the hydrophilicity of the conjugate.

In another aspect of the invention, a proline or alanine is added to the N-terminus of the peptide. In a preferred aspect, a proline or alanine is added to the N-terminus of an enkephalin peptide and the oligomer moiety is coupled to the N-terminus of the proline or alanine residue.

After absorption into the central nervous system, the esters of the fatty sugar are hydrolyzed leaving a hydrophilic moiety. Efflux is hindered and the brain aminopeptidases cleave the proline or alanine portion leaving the peptide to regain full activity.

The invention also provides a pharmaceutical composition comprising an amphiphilic drug-oligomer conjugate and a pharmaceutically acceptable carrier.

In another aspect, a pharmaceutical composition is provided comprising (1) a mixture of an enkephalin conjugate according to the present invention wherein the enkephalin peptide has proline or alanine added to its N-terminus and an enkephalin conjugate according to the present invention which does not have a proline or alanine added to the N-terminus, and (2) a pharmaceutical carrier. This aspect provides a faster acting sustained dose of enkephalin.

The invention also provides methods of administering a conjugate of the invention.

The invention further provides assays, both in vitro and in vivo, for testing the efficacy of the conjugates of the invention.

Other objects and further scope of applicability of the present invention will become apparent from the detailed description given hereafter. It should be understood that the

detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications will become apparent to those skilled in the art from the detailed description.

5 2.1 DEFINITIONS

As used herein, the term "lipophilic" means the ability to dissolve in lipids and/or the ability to penetrate, interact with and/or traverse biological membranes.

As used herein, the term, "lipophilic moiety" or "lipophile" means a moiety which is lipophilic and/or which, when attached to another chemical entity, increases the lipophilicity of such chemical entity, e.g., fatty acid, cholesterol.

As used herein, the term "hydrophilic" means the ability to dissolve in water.

As used herein, the term "hydrophilic moiety" or "hydrophile" refers to a moiety which is hydrophilic and/or which when attached to another chemical entity, increases the hydrophilicity of such chemical entity, e.g., sugars, PEG.

As used herein, the term "amphiphilic" means the ability to dissolve in both water and lipids.

As used herein, the term "amphiphilic moiety" means a moiety which is amphiphilic and/or which when attached to a peptide or non-peptide drug increases the amphiphilicity of the resulting conjugate, e.g., PEG-fatty acid oligomer, sugar-fatty acid oligomer.

As used herewith, the term "neuroactive drug" is used broadly to encompass any peptide or other drug having an activity within the CNS, e.g., enkephalin, enkephalin analogues.

As used herein the term "peptide" is intended to be broadly construed as inclusive of polypeptides per se having molecular weights of up to about 10,000, as well as proteins having molecular weights of greater than about 10,000.

As used herein, the term "covalently coupled" means that the specified moieties are either directly covalently bonded to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

- 5 As used herein, the term "drug" means a substance intended for use in the diagnosis, characterization, cure, mitigation, treatment, prevention or allaying the onset of a disease, disease state, or other physiological condition or to enhance normal physiological functioning in humans and/or in non-human animals.

3. BRIEF DESCRIPTION OF THE FIGURES

3.1 FIGURES 1A-1C: Formulae 8-10; amphiphilic oligomers of the present invention where in the lipophile is a sugar. In 1b and 1c, PEG is used as a spacer group. In 1A-1C a proline residue is added at the N-terminus of the enkephalin peptide.

5 3.2 FIGURE 2: Compares the stability of the cetyl-PEG₂-enkephalin-lys conjugate (non-hydrolyzable) to unconjugated enkephalin in rat brain homogenate.

3.3 FIGURE 3: Compares the stability of the cetyl-PEG₃-enkephalin conjugate (non-hydrolyzable) to unconjugated enkephalin in rat brain homogenate.

10 3.4 FIGURE 4: Compares palmitate-PEG₃-Enk conjugate (hydrolyzable) to unconjugated enkephalin in rat brain homogenate.

3.5 FIGURE 5a-5d: HPLC data showing extraction of conjugate from homogenized rat brain.

3.6 FIGURE 6: Graph demonstrating competitive binding between cetyl-PEG₂-enkephalin conjugate and naloxone, an Opioid μ receptor agonist.

15 3.7 FIGURE 7: Graphic comparison of analgesic effect of cetyl-PEG₂-enkephalin with clonidine (a morphine substitute).

3.8 FIGURE 8: Table showing results of receptor binding assays for various conjugates according to the present invention.

20 3.9 FIGURE 9: Exemplary synthetic scheme for an oligomer according to the present invention.

3.10 FIGURE 10: Exemplary synthetic scheme showing attachment of an oligomer to an enkephalin peptide according to the present invention.

4. DETAILED DESCRIPTION OF THE INVENTION

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

The present invention relates generally to amphiphilic drug-oligomer conjugates capable of traversing the BBB and to methods of making and using such conjugates.

- 5 The drugs are preferably neuro-active drugs, proteins, peptides and especially enkephalin analogues. The conjugates are stable in the environment of the bloodstream and resist degradation by the BBB. The conjugates readily traverse the BBB.

10 In one aspect, the conjugates produce their intended pharmacological effect without requiring metabolic cleavage of the oligomer. When cleavage of the oligomer occurs, the drug retains activity.

15 The amphiphilic oligomers are composed of lipophilic and hydrophilic moieties. The lipophilic moieties are preferably natural fatty acids or alkyl chains. The lipophilic moieties are preferably small segments of PEG, having 1 to 7 PEG moieties, and preferably having 1 to 5 PEG moieties. The length and composition of the lipophilic moieties and the hydrophilic moieties may be adjusted to obtain desired amphiphilicity. For example, the carbon chains of the fatty acid or alkyl moieties may be lengthened to increase lipophilicity, while PEG moieties may be lengthened to increase hydrophilicity.

20 Preferably, the fatty-acid moiety is a straight chain molecule having saturated and unsaturated carbons and ranges from four (4) to twenty-six (26) carbon atoms. Most preferably, the fatty acid has from fourteen (14) to twenty-two (22) carbon atoms.

A cholesterol or adamantane moiety can be substituted for straight chain fatty acid as the lipophilic portion of the oligomers.

Examples of preferred oligomers are as follows:



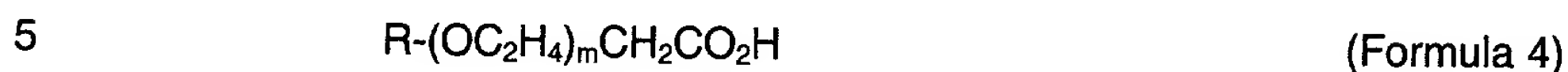
25 wherein $n=3$ to 25 and $m=1$ to 7;



wherein $n=3$ to 25 and $m=1$ to 6;



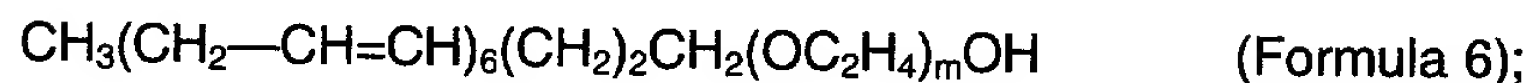
wherein $n=3$ to 25, and $m=1$ to 7;



wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 5;



10 wherein $m=1$ to 7;



wherein $m=1$ to 7.

Other unsaturated fatty acid moieties which can be used according to the present invention include oleic, linoleic and linolenic.

- 15 In certain instances, it is preferred to provide hydrolyzable bonds between the polyethylene glycol and the fatty acid moieties. This permits hydrolysis to occur after penetration into the central nervous system, thus releasing the active peptides with the polyethylene glycol group still attached to the peptide. The peptides acquire a more hydrophilic character and efflux to circulatory system is thereby hindered.

The covalent bond between the oligomer and the drug is preferably amide (a carboxy group of the oligomer is linked to an amine group of the peptide), or carbamate (an chloroformate group of the oligomer is linked to an amine group of the peptide).

- 5 For non-peptide drug, the bond is preferably ester (a carboxy group of the peptide is covalently coupled to a hydroxyl group of the oligomer or a carboxy group of the oligomer is covalently coupled to a hydroxyl group of the drug), amide (a carboxy group of the oligomer is linked to an amine group of the drug) or carbamate (a chloroformate group of the oligomer is linked to an amine group of the drug). For the enkephalin analogues, the preferred peptides are leu-enkephalin lysine and met-enkephalin lysine.
- 10 The amino residue of the lysine is preferably utilized in bonding.

Other preferred amphiphilic moieties are sugar moieties, coupled to natural fatty acids and segments of polyethylene glycol. The PEG moiety serves to increase the amphiphilicity of the fatty sugar.

- 15 The length and number of the PEG moieties can be varied to refine the amphiphilicity of the conjugate. Increasing the number of PEGs increases the hydrophilicity of the resulting oligomer.

- In certain instances, it is preferred to modify the N-terminus of an enkephalin with proline or alanine before attaching the oligomer. After absorption into the central nervous system, the esters of the fatty sugar are hydrolyzed leaving hydrophilic moiety. Easy
- 20 efflux is hindered and the brain aminopeptidases cleave the proline or alanine portion leaving the peptide to regain full activity.

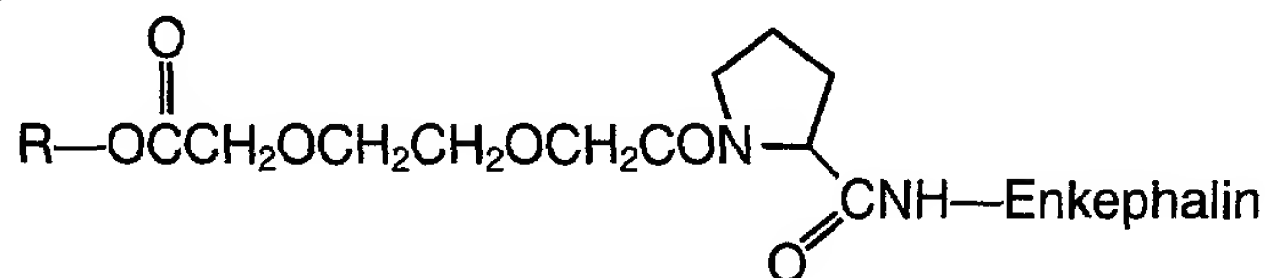
Where the hydrophilic moiety is a sugar, it is preferred that the sugar is a monosaccharide. The sugar may be an amino sugar or a non-amino sugar.

- 25 In another aspect, the oligomer is attached to the C-terminus of the peptide drug. For example:

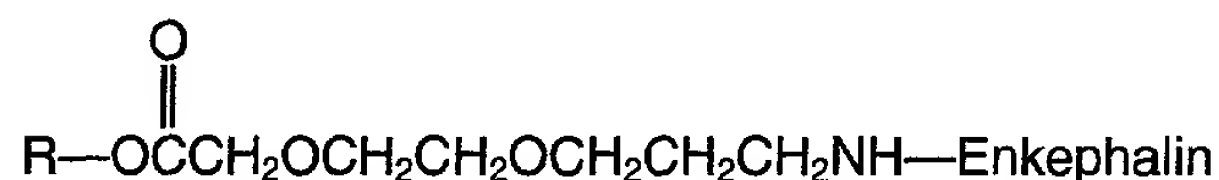


Wherein R=alkyl₁₋₂₆, cholesterol or amantane.

In another aspect, the oligomer is attached at the N-terminus of the peptide drug. For example:



5



It will be appreciated by one of skill in the art that the oligomers may be attached at the carboxy terminus or at a constituent of an amino acid side chain, such as at the amino group of lysine.

- 10 The present invention broadly relates to therapeutic and/or diagnostic conjugates wherein the therapeutic and/or diagnostic molecule is covalently bonded to an oligomer to form an amphiphilic conjugate. In one aspect, the oligomer comprises at least one lipophilic moiety and at least one hydrophilic moiety, and the size and nature of the two moieties is so selected as to impart an amphiphilic nature to the resulting conjugate.

- 15 Exemplary oligomers according to the present invention are as follows:



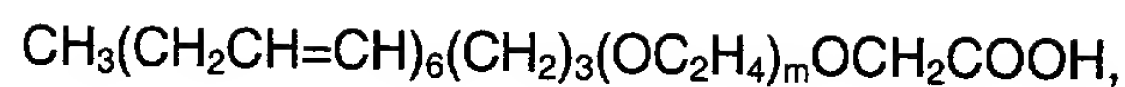
where m=1 to 7;



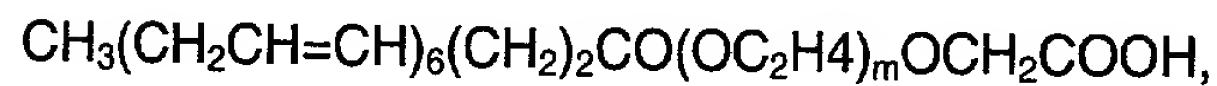
where m=1 to 7;

- 20 $\text{CH}_3(\text{CH}_2\text{CH}=\text{CH})_6(\text{CH}_2)_2\text{CONHCH}_2\text{CH}_2(\text{OC}_2\text{H}_4)_m\text{OH},$

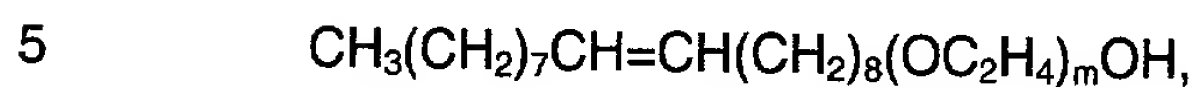
where m=1 to 6;



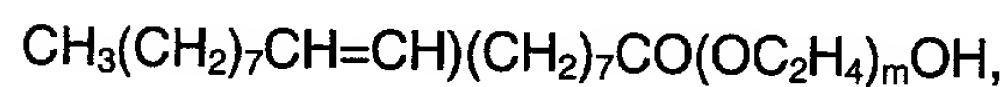
where $m=1$ to 6;



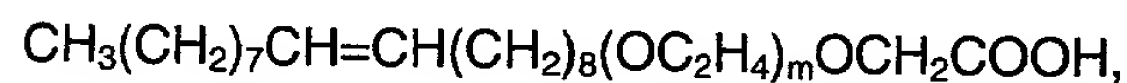
where $m=1$ to 6;



where $m=1$ to 7;



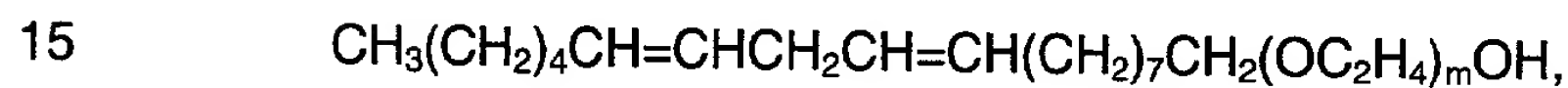
where $m=1$ to 7;



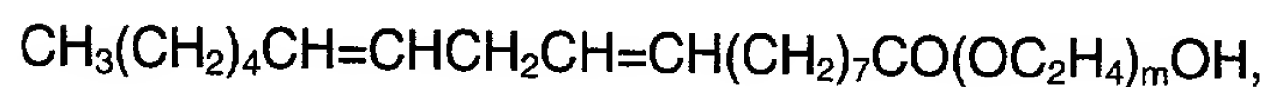
where $m=1$ to 6;



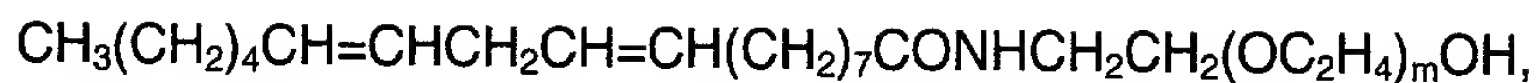
where $m=1$ to 6;



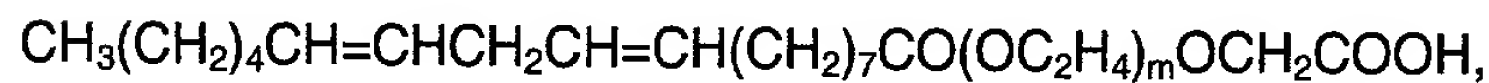
where $m=1$ to 6;



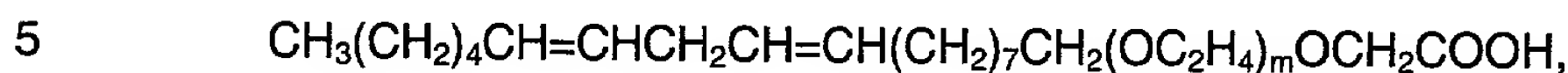
where $m=1$ to 7;



where $m=1$ to 6;



where $m=1$ to 6;



where $m=1$ to 6;



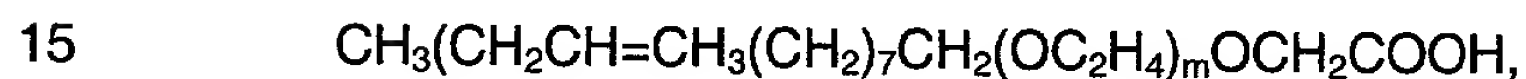
where $m=1$ to 7;



where $m=1$ to 6;



where $m=1$ to 6;



where $m=1$ to 6.

4.1 THERAPEUTIC COMPOUNDS

The invention thus comprehends various compositions for therapeutic (in vivo) application, wherein the peptide component of the conjugated peptide complex is a physiologically active, or bioactive, peptide. In such peptide-containing compositions, the conjugation of the peptide component to the oligomer may be by direct covalent bonding or indirect (through appropriate spacer groups) bonding, and the hydrophilic and lipophilic moieties may also be structurally arranged in the oligomer in any suitable manner involving direct or indirect covalent bonding, relative to one another. A wide variety of peptide species may be accommodated in the broad practice of the present invention, as necessary or desirable in a given end use therapeutic application.

While the description is primarily and illustratively directed to the use of enkephalin as a peptide component in various compositions and formulations of the invention, it will be appreciated that the utility of the invention is not thus limited, but rather extends to any peptide species which capable of conjugation to the oligomers herein described, or which are capable of being modified, as for example by the incorporation of a proline residue, so as to enable the peptide to be conjugated to the oligomers described herein.

Accordingly, appropriate peptides include, but or not limited to: adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminase, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystikinin, clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen activator, trypsin, vasopressin, and analogues of such peptides, as well as other suitable enzymes, hormones, proteins, polypeptides, enzyme-protein conjugates, antibody-hapten conjugates, viral epitopes, etc.

In other aspect, the therapeutic peptide of the amphiphilic drug-oligomer conjugates are as described in United States Patent 5,641,861, which is incorporated herein by reference, so long as any of such peptides contains a lysine residue. Exemplary peptides described therein include: Ac-Phe-Arg-Trp-Trp-Tyr-Lys—NH₂; Ac-Arg-Trp-Ile-

Gly-Trp-Lys—NH₂; Trp-Pro-Lys-His-Xaa—NH₂, where Xaa can be any one of the twenty naturally occurring amino acids, or Trp-Trp-Pro-Xaa—NH₂, where Xaa is Lys or Arg; Tyr-Pro-Phe-Gly-Phe-Xaa—NH₂, wherein Xaa can be any one of the twenty naturally occurring amino acids; (D)Ile-(D)Met-(D)Ser-(D)Trp-(D)Trp-Gly_n-Xaa—NH₂, wherein Xaa is Gly or the D-form of a naturally-occurring amino acid and n is 0 or 1, peptides of this formula can be hexapeptides when Gly is absent (n is 0) and heptapeptides when Gly is present (n is 1); (D)Ile-(D)Met-(D)Thr-(D)Trp-Gly-Xaa—NH₂, wherein Xaa is Gly or the D-form of a naturally-occurring amino acid; Tyr-A1-B2-C3—NH₂, wherein A1 is (D)Nve or (D)Nle, B2 is Gly, Phe, or Trp, and C3 is Trp or Nap; Pm and red {Me_xH_yN-Tyr-(NMe)_z-Tyr-Xaa_z—NH₂}, wherein x and y independently are 0, 1, or 2 and z is 0 or 1, and wherein Xaa is Phe D-Phe, or NHBzl.

In other aspect, the therapeutic peptide of the amphiphilic drug-oligomer conjugates are as described in United States Patent 5,602,099, which is incorporated herein by reference. with the proviso that the conjugation can occur only where there is a free carboxyl or free N-terminal. Exemplary peptides include: H-Tyr-Tic-Phe-Phe-OH; H-Tyr-Tic-Phe-Phe-NH₂; Tyr(Nα Me)-Tic-Phe-Phe-OH; Tyr(Nα Cpm)-Tic-Phe-Phe-OH; Tyr(Nα Hex)-Tic-Phe-Phe-OH; Tyr(Nα Et₂)-Tic-Phe-Phe-OH; H-Dmt-Tic-Phe-Phe-OH; H-Dmt-Tic-Phe-Phe-NH₂; H-Tyr(3-F)-Tic-Phe-Phe-OH; H-Tyr(3-Cl)-Tic-Phe-Phe-OH; H-Tyr(3-Br)-Tic-Phe-Phe-OH; H-Dmt-TicΨ [CH₂—NH]Phe-Phe-OH; H-Dmt-TicΨ [CH₂—NH]Phe-Phe-NH₂; H-Tyr-TicΨ [CH₂—NCH₃]Phe-Phe-OH; H-Tyr-Tic-Ψ [CH₂—NH]Hfe-Phe-OH; Tyr(NMe)-TicΨ [CH₂—NH]Hfe-Phe-OH); H-Tyr-Tic-Phg-Phe-OH; H-Tyr-Tic-Trp-Phe-OH; H-Tyr-Tic-Trp-Phe-NH₂; H-Tyr-Tic-His-Phe-OH; H-Tyr-Tic-2-Nal-Phe-OH; H-Tyr-Tic-Atc-Phe-OH; H-Tyr-Tic-Phe-Phe(pNO₂)-OH; H-Tyr-Tic-Trp-Phe(pNO₂)-OH; H-Tyr-Tic-Phe-Trp-NH₂; H-Tyr-Tic-Phe-Phe-Val-Val-Gly-NH₂; H-Tyr-Tic-Phe-Phe-Tyr-Pro-Ser-NH₂; H-Tyr-Tic-Trp-Phe-Tyr-Pro-Ser-NH₂; H-Tyr-Tic-Trp-Phe (pNO₂) -Tyr-Pro-Ser-NH₂ and H-Tyr-Tic-Phe-Phe-Leu-Nle-Asp-NH₂.

Abbreviations in the aforementioned peptides of U.S. Patent 5,602,099 may be interpreted as follows: Aib=α-aminoisobutyric acid; Atc=2-aminotetralin-2-carboxylic acid; Boc=tert-butoxycarbonyl; Cpm=cyclopropylmethyl; DCC=dicyclohexyl-

carbodiimide; DIEA=diisopropylethylamine; Dmt=2,6-dimethyltyrosine; Et=ethyl; Hex=hexyl; Hfe=homophenylalanine; HOBt=1-hydroxybenzotriazole; MVD=mouse vas deferens; 1-Nal=3-(1'-naphthyl)alanine; 2-Nal=3-(2'-naphthyl)alanine; Phe(pNO₂)=4-nitrophenylalanine; Phg=phenylglycine; Tic=1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP=H-Tyr-Tic-Phe-OH; TIP-NH₂=H-Tyr-Tic-Phe-NH₂; TIP(Ψ)=H-Tyr-TicΨ [CH₂-NH]Phe-OH; TIPP=H-Tyr-Tic-Phe-Phe-OH; TIPP-NH₂=H-Tyr-Tic-Phe-Phe-NH₂; TIPP(Ψ)=H-Tyr-TicΨ [CH₂-NH]Phe-Phe-OH; Tyr(3-Br)=3-bromotyrosine; Tyr(3-Cl)=3-chlorotyrosine; Tyr(3-F)=3-fluorotyrosine; and Tyr(Nα Me)=Nα-methyltyrosine.

In another aspect, the peptides are as described in U.S. Patent 5,545,719, which is incorporated herein by reference.

Other exemplary peptides include, for example, ACTH-related peptides for inducing neural regeneration, cyclosporin for treating infection, enkephalin analogs for treating pain and drug addiction, MIF-1 for treating depression, neurotensin for relieving pain, and peptide T for treating AIDS-associated dementia. Adrenocorticotrophic hormone (ACTH) and its analogue peptides are also known to restore the avoidance learning caused by removal of the pituitary gland and can also be used to treat passive avoidance conditions.

Particularly preferred peptides are the endogenous and synthetic Opioid peptides such as the enkephalins. A particularly preferred Opioid is [Met⁵]Enkephalin (Tyr-Gly-Gly-Phe-Met).

Peptides according to the present invention may be synthesized according to any method of synthesis known in the art. Such methods include, but are not limited to chemical synthesis techniques and recombinant DNA expression techniques.

The therapeutic compounds of the present invention can be modified in order to facilitate coupling to the amphiphilic oligomer. A functional group may be added to the C-terminus or the N-terminus of the peptide or to a side chain of the peptide in order to provide a point of attachment for the oligomer.

Alternatively, specific amino acids may be inserted within the amino acid chain of the peptide therapeutic, or may replace an amino acid of the therapeutic or may be added to the C-terminus or N-terminus of the peptide in order to facilitate attachment of the oligomer where such modification does not eliminate the activity of the peptide. For example, a proline or alanine residue can be added to the N-terminus of a therapeutic peptide, such as an enkephalin, such as [met⁵]enkephalin, in order to facilitate attachment of the amphiphilic oligomer.

One skilled in the art would know that one or more amino acids within the exemplified peptides could be modified or substituted, as for example, by a conservative amino acid substitution of one or more of the specific amino acids shown in the exemplified peptides. A conservative amino acid substitution change can include, for example, the substitution of one acidic amino acid for another acidic amino acid, of one hydrophobic amino acid for another hydrophobic amino acid or other conservative substitutions known in the art, including the use of non-naturally occurring amino acids, such as Nle for Leu or ornithine (Orn) or homoArginine (homoArg) for Arg.

In addition to the above types of modifications or substitutions, a mimic of one or more amino acids, otherwise known as a peptide mimetic or peptidomimetic, can also be used. As used herein, the term "mimic" means an amino acid or an amino acid analog that has the same or similar functional characteristic of an amino acid. Thus, for example, a (D)arginine analog can be a mimic of (D)arginine if the analog contains a side chain having a positive charge at physiological pH, as is characteristic of the guanidinium side chain reactive group of arginine. A peptide mimetic or peptidomimetic is an organic molecule that retains similar peptide chain pharmacophore groups as are present in the corresponding peptide.

The substitution of amino acids by non-naturally occurring amino acids and peptidomimetics as described above can enhance the overall activity or properties of an individual peptide based on the modifications to the side chain functionalities. For example, these types of alterations can be employed along with the amphiphilic oligomers of the present invention to further enhance the peptide's stability to enzymatic breakdown and increase biological activity.

One skilled in the art can easily synthesize the peptides for use as therapeutics in this invention. Standard procedures for preparing synthetic peptides are well known in the art. The peptides can be synthesized using the solid phase peptide synthesis (SPPS) method of Merrifield (*J. Am. Chem. Soc.*, 85:2149 (1964), which is incorporated herein by reference) or using standard solution methods well known in the art (see, for example, Bodanzsky, M., *Principles of Peptide Synthesis* 2nd revised ed. (Springer-Verlag, 1988 and 1993), which is incorporated herein by reference). Alternatively, simultaneous multiple peptide synthesis (SMPS) techniques well known in the art can be used. Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, Calif.) or using the manual peptide synthesis technique described by Houghten, *Proc. Natl. Acad. Sci., USA* 82:5131 (1985), which is incorporated herein by reference.

Peptides can be synthesized using amino acids or amino acid analogs, the active groups of which are protected as necessary using, for example, a t-butyldicarbonate (t-BOC) group or a fluorenylmethoxy carbonyl (Fmoc) group. Amino acids and amino acid analogs can be purchased commercially (Sigma Chemical Co.; Advanced Chemtec) or synthesized using methods known in the art. Peptides synthesized using the solid phase method can be attached to resins including 4-methylbenzhydrylamine (MBHA), 4-(oxymethyl)-phenylacetamidomethyl and 4-(hydroxymethyl)phenoxymethyl-copoly(styrene-1% divinylbenzene) (Wang resin), all of which are commercially available, or to p-nitrobenzophenone oxime polymer (oxime resin), which can be synthesized as described by De Grado and Kaiser, *J. Org. Chem.* 47:3258 (1982), which is incorporated herein by reference.

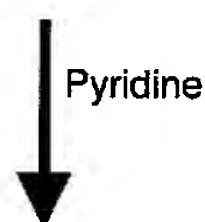
A newly synthesized peptide can be purified using a method such as reverse phase high performance liquid chromatography (RP-HPLC) or other methods of separation based on the size or charge of the peptide. Furthermore, the purified peptide can be characterized using these and other well known methods such as amino acid analysis and mass spectrometry.

4.2 SYNTHESIS

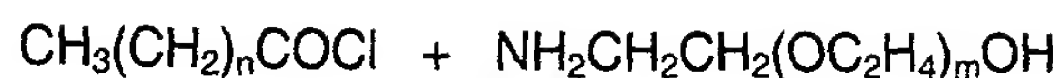
A general synthesis scheme for the oligomers of the present invention is provided in FIG. 9, and a general synthesis scheme for attaching such oligomer to the therapeutic peptides of the instant invention is provided in FIG. 10.

Several methods of modifying fatty acid to achieve the desired oligomer will be discussed in further detail with structural illustrations.

In the synthesis of oligomers containing fatty acids and polyethylene glycols, where the ethylene glycol is connected to the fatty acid in a hydrolysable ester bond, it is desirable to start with the acid chloride of the fatty acid or its acid anhydride. A desired polyethylene glycol having two free hydroxyls at the termini is then treated in inert solvent with equal molar equivalent of acid chloride or acid anhydride. The glycol unit is first dissolved in inert solvent and treated with organic base before the addition of the acid chloride or acid anhydride. The product is extracted from the reaction medium and further purified using column chromatograph:



In some instances it is desired to create oligomers that have stronger hydrolysable bond such as amide. The acid chloride or the acid anhydride of the selected fatty acid is treated with amino derivative of polyethylene glycol in a controlled reaction condition to effect only the amino residue and not the hydroxyl portion. Other conditions that ensure selectivity is by converting the fatty acid into N-hydroxysuccinimide ester and reacting with the amino residue of the polyethylene glycol.



↓
Pyridine



5



↓
THF/TEA

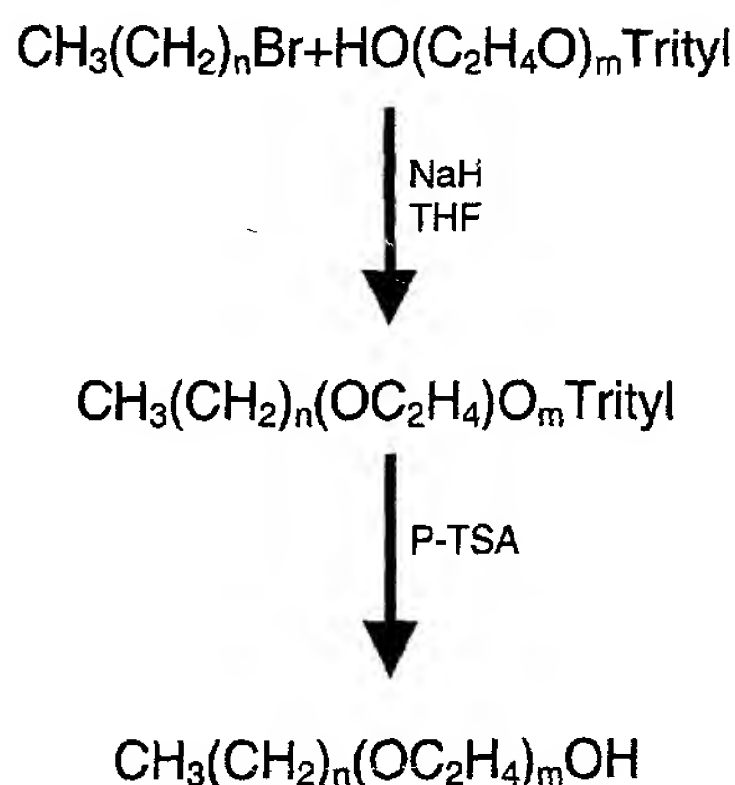


- 10 Coupling of the oligomer to the peptide drug is effected by converting the free hydroxyl moiety of the oligomer to N-hydroxysuccinimide ester (NSU). N-hydroxysuccinimide group reacts readily with the nucleophilic amino residue of the peptide.



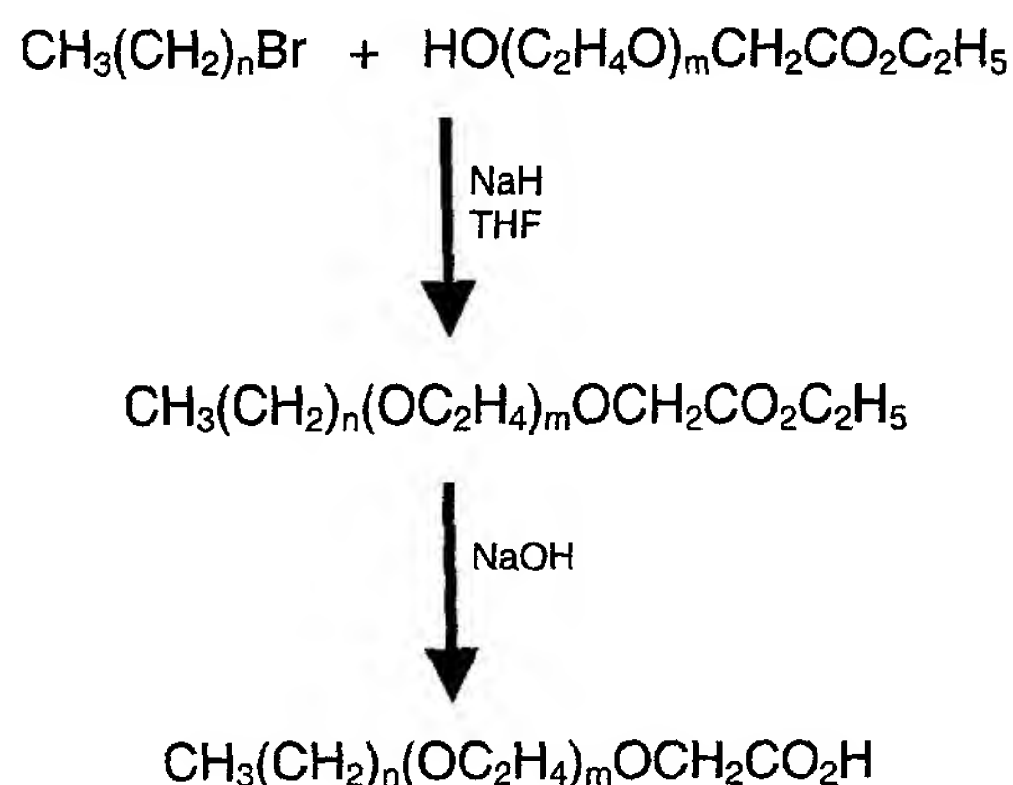
- 15 In the synthesis of oligomers in which the lipophilic portion of the oligomers is connected to the hydrophilic portion by ether linkage, the desired polyethylene glycol (hydrophile) is first protected. One of the two free hydroxyls at the termini is protected with a trityl group in pyridine using one mole of trityl chloride. The protected polyethylene glycol is dissolved in a suitable inert solvent and treated with sodium hydride. Bromo or tosylate derivative of the lipophilic portion is dissolved in inert solvent and added to the solution of the protected polyethylene glycol. The product is treated with a solution of para-toluenesulfonic acid in anhydrous inert solvent at room temperature. The desired
- 20

product is extracted in inert solvent and purified by column chromatography. The structures of the transformation are depicted below:



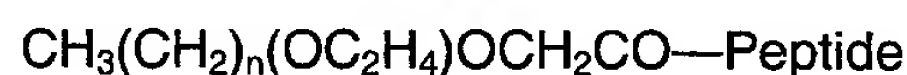
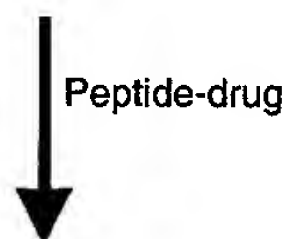
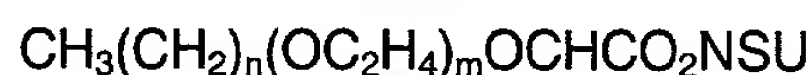
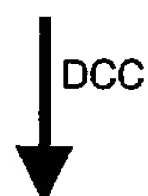
The lipophilic portion can be alkyl, cholesteryl, adamantyl moieties.

In the synthesis of oligomers where the lipophilic portion of the oligomer is connected to the hydrophilic portion in ether bond and the terminal ends in carboxylic acid moiety, it is desirable to protect the carboxylic group. Polyethylene glycol having free hydroxyl group at one end and carboxylic group at the other end is selected. The carboxylic group is protected by esterification. The protected polyethylene glycol is dissolved in a suitable inert solvent and treated with sodium hydride. Bromo or tosylate derivatives of the lipophilic portion is dissolved in inert solvent and added to the solution of the protected polyethylene glycol. The product is treated with solution of sodium hydroxide to liberate free acid. The desired product is extracted in inert solvent and purified by column chromatography. The structures of the transformation are depicted below.



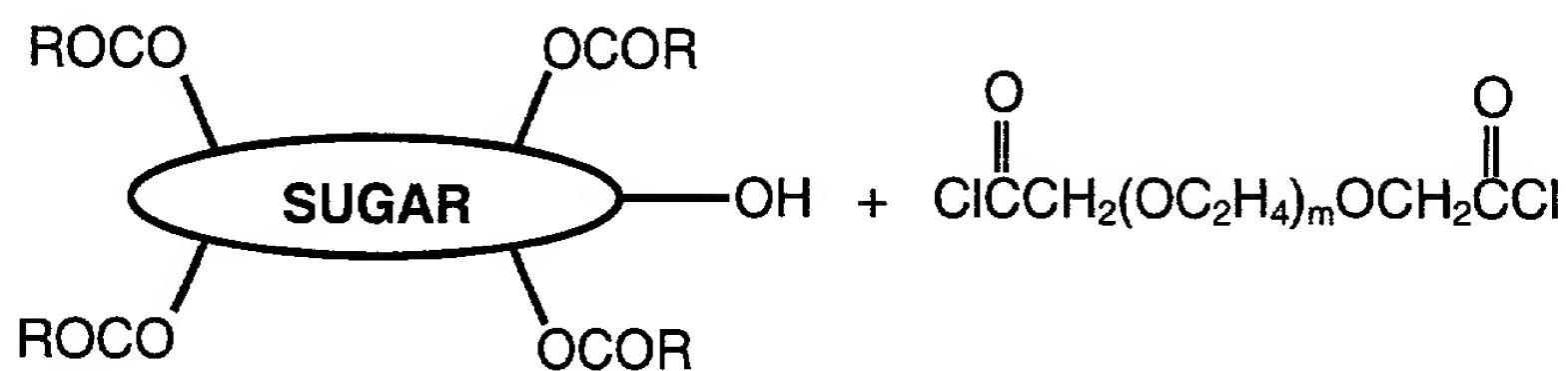
The lipophilic portion can be alkyl, cholesteryl or adamantyl moieties.

This group of acidic oligomers can be coupled to peptide drugs by first reacting the carboxylic group with N-hydroxysuccinimide (NSU) to form easily leavable group. A solution of the activated oligomers in inert solvent is treated with the desired peptide drug dissolved in a suitable solvent. Inverse addition may be selected.



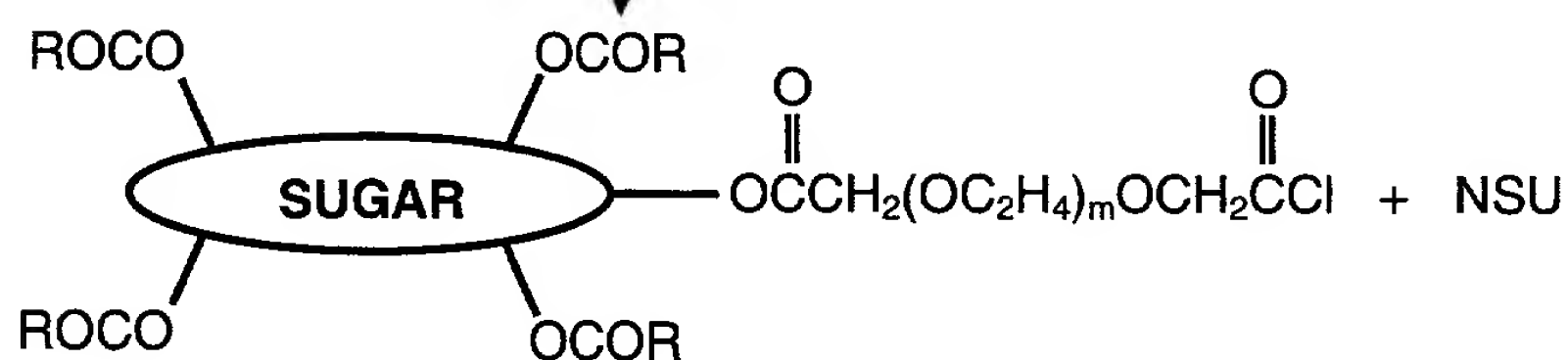
Sometimes it is desirable to replace the lipophilic moiety with lipophilic sugars. The sugar moiety is first esterified with desired fatty acid chloride to obtain selective or partial acylation. The product is treated in inert solvent with diacid chloride of desired dicarboxylic acid derivative of polyethylene glycol.

Reaction is conducted with one molar equivalent of each reacting moiety. This reaction leaves one end of the hydrophile bearing acid chloride, which is further converted to N-hydroxysuccinimide ester. The activated ester is reacted with peptide drug in suitable inert solvent.



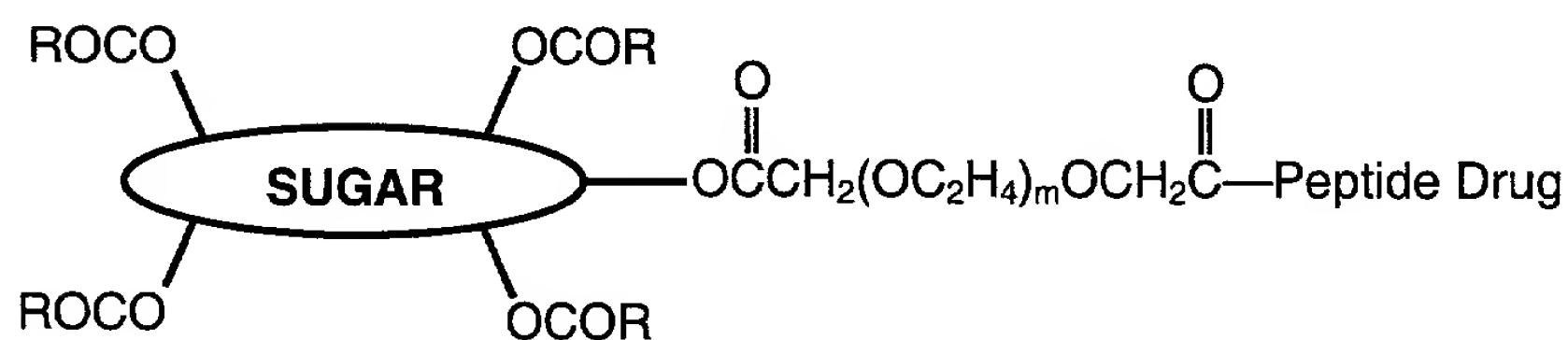
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Pyridine



10

Peptide Drug



15 Where R=fatty acid, alkyl₁₋₂₆, cholesterol or adamantane.

4.3 THERAPEUTIC METHODS

The invention provides methods of treatment and prevention by administration to a subject of an effective amount of an amphiphilic drug-oligomer conjugate of the invention.

One embodiment of the invention provides for methods of administering a pharmaceutical composition which is comprised of a therapeutically effective amount of an amphiphilic drug-oligomer conjugate according to the present invention.

Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The conjugates may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

In certain circumstances, it may be desirable to introduce the pharmaceutical compositions of the invention directly into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Pulmonary or nasal administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In another embodiment, the conjugates can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

The subject is preferably an animal, including, but not limited to, animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

4.4 PHARMACEUTICAL COMPOSITIONS

Exemplary means of administration include oral, parenteral, rectal, topical, sublingual, mucosal, nasal, ophthalmic, subcutaneous, intramuscular, intravenous, transdermal, spinal, intrathecal, intra-articular, intra-arterial, sub-arachnoid, bronchial, lymphatic, and intrauterine administration.

The present invention contemplates the use of pharmaceutical formulations for human medical use which comprise the vector structures of the present invention as therapeutic ingredients. Such pharmaceutical formulations may include pharmaceutically effective carriers, and optionally, may include other therapeutic ingredients. The carrier or carriers must be pharmaceutically acceptable in the sense that they are compatible with the therapeutic ingredients and are not unduly deleterious to the recipient thereof. The therapeutic ingredient or ingredients are provided in an amount necessary to achieve the desired therapeutic effect, described below.

In another aspect, a pharmaceutical composition is provided to comprising (1) a mixture of an enkephalin conjugate according to the present invention wherein the enkephalin peptide has proline or alanine added to its N-terminus and an enkephalin conjugate according to the present invention which does not have a proline or alanine added to the N-terminus, and (2) a pharmaceutical carrier.

Various delivery systems are known and can be used to administer a conjugate of the invention, e.g., encapsulation microcapsules.

As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the conjugate is administered.

Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean

oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

- 5 Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

- 10 The compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

- 15 Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

- 20 Such compositions will contain a therapeutically effective amount of the drug-oligomer conjugate, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

- 25 The mode of administration and dosage forms will of course affect the therapeutic amounts of the compounds which are desirable and efficacious for the given treatment application. A therapeutically effective amount is an amount necessary to prevent, delay or reduce the severity of the onset of disease, or an amount necessary to arrest or reduce the severity of an ongoing disease. It will be readily apparent to one of skill in the art that this amount will vary based on factors such as the weight and health of the

recipient, the type of cells being transformed, the mode of administration of the present compositions and the type of medical disorder being treated.

The dosage can be presented in the form of tablets, syrups, lozenges, elixirs, suspensions, and/or emulsions.

- 5 Accessory ingredients may, without limitation, include diluents, buffers, flavoring agents, disintegrants, surfactants, thickeners, lubricants, preservatives, and/or antioxidants.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings.

- 10 Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a
15 hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent.

- Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or
20 saline can be provided so that the ingredients may be mixed prior to administration.

The conjugates of the invention can be formulated as neutral or salt forms.

- Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium,
25 ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Conjugate of the invention which will be therapeutically effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vivo* and/or *in vitro* assays may optionally be employed to help identify optimal dosage ranges.

For example, suitable doses of a an enkephalin conjugate for analgesia may genarally be in the range of from 1 mg/kg to 20 mg/kg, preferably 3 mg/kg to 15 mg/kg, more preferably 5 mg/kg to 7 mg/kg.

Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

5. EXAMPLES

5.1 SYNTHESIS

5.1.1 TRIETHYLENE GLYCOL MONOHEXADECYL ESTER

Palmitic anhydride (5.00g; 10.104 mmol) was dissolved in dry THF (20 mL) and 3 mol excess of dry pyridine and the solution was stirred at room temperature. To the stirring solution, triethylene glycol (1.5 g; 10.104 mmol) was added slowly. After stirring for 1h, THF was removed under reduced pressure at room temperature and the reaction mixture was poured into ice cold 10% sulfuric acid. The aqueous layer was extracted with ethyl acetate (30 ml x 3). Combined organic layer was sequentially washed with water, brine and dried over MgSO_4 and filtered. After evaporation gave pure product, single spot on TLC.

5.1.2 SUCCINIMIDYL TRIETHYLENE GLYCOL MONOHEXADECYL ESTER:

To a stirring solution of teg-palmitate (1 g; 2.57 mmol), dimethylaminopyridine (0.313 g; 2.57 mmol) in dry THF was added N,N'-disuccinimidyl carbonate (0.691 g) in one portion. The reaction mixture was stirred overnight at room temperature. The organic solvent was removed under reduced pressure and reaction mixture was diluted with ethyl acetate, washed with 1N hydrochloric acid (10mL x 2), water and brine. The solvent was dried over MgSO_4 , filtered and evaporated to leave white solid.

5.1.2.1 Determination of Activity

The succinimidyl reactivity was determined by conjugating it with insulin and it was found to be 67%.

5.1.3 SUCCINIMIDYL TRIETHYLENE GLYCOL MONOHEXADECYL ETHER

To a cold stirring solution of phosgene (10.0 mL; 20% solution in toluene) under nitrogen, a solution of triethylene glycol monohexadecyl ether (1.5 g; 4.00 mmol) in dry dichloromethane (4mL) was added. The reaction mixture was stirred at 0 °C for 2h at room temperature. Excess of phosgene was distilled off using water aspirator, passing through cold solution of dilute NaOH.

The reaction flask was cooled in ice bath and equimolar quantity of triethyl amine and a solution of hydroxysuccinimide, dissolved in minimum quantity of THF was added slowly. The reaction mixture was stirred at room temperature for 12h. The solvent was removed

completely at 25 °C and residue was redissolved in ethyl acetate, washed with water, brine, dried over MgSO₄ and evaporated to give pure succinimidyl derivative

5.1.3.1 DETERMINATION OF ACTIVITY

The succinimidyl reactivity was determined by conjugating it with insulin and it was found to be 62.5%.

5.2 CONJUGATION OF COMPOUND 2 & 4 WITH MET-ENKEPHLIN

5.2.1 GENERAL PROCEDURE FOR CONJUGATION

5.2.1.1 SCHEME



5.2.1.2 GENERAL PROCEDURE

To a stirring solution of met-enkephalin (0.130 g; 0.1854 mmol) in 5mL of DMF-DCM (2:1) was added TEA (25 µL). The reaction mixture was cooled to 10 °C and a solution of palmityl-teg-nsu or cetyl-teg-nsu dissolved in 1mL of DCM was added in one portion. The reaction mixture was stirred for 2h at 10 °C. The solvent was removed under reduced pressure and the residue was redissolved in dry ethyl acetate. After evaporation of the solvent 0.310g conjugated enkephalin was obtained. HPLC showed mono & diconjugate in the ratio of 3.1.

5.3 SYNTHESIS OF CETYL-PEG₂; IT'S ACTIVATION & CONJUGATION WITH PROTECTED (BOC) LEUENK

To a suspension of NaH (4.00g; 0.1 mol) in dry THF (300 mL) at 10 °C was added diethylene glycol in one portion. The cooling bath was removed and reaction mixture was stirred at room temperature for 2h. At the end the reaction mixture was cooled to 10 °C and bromohexadecane (29g).095 mol) was added in one portion. The cooling bath was removed and the reaction was stirred at room temperature for 4h. The solvent was removed under reduced pressure and crude was admixed with water and extracted with ethyl acetate (30 mL x 3). The combined organic extract was sequentially washed with water, brine, dried over MgSO₄ and evaporated to leave white solid powder, single spot on TLC and single molecular ion peak.

5.3.1 CETYL-PEG₂-NSU

To a cold stirring solution of phosgene (10.0mL; 20% solution in toluene) under nitrogen, a solution of cetyl-PEG₂-OH (1.3g; 4.00 mmol) in dry dichloromethane (5mL) was added. The reaction mixture was stirred at 0 °C for 1hr and 2h at room temperature. Excess of phosgene was distilled off using water aspirator, passing through cold solution of dilute NaOH.

The reaction flask was cooled in ice bath and equimolar quantity of triethyl amine and a solution of hydroxy succinimide, dissolved in minimum quantity of THF was added slowly. The reaction mixture was stirred at room temperature for 12h. The solvent was removed completely at 25 °C and residue was redissolved in ethyl acetate, washed with water, brine, dried over MgSO₄ and evaporated to give pure succinimidyl derivative.

5.3.2 DETERMINATION OF ACTIVITY

The succinimidyl reactivity was determined by conjugating it with insulin and it was found to be 83.5%.

5.4 CONJUGATION OF SUCCINIMIDYL CETYL-PEG₂ WITH BOC-LEU... ENK... LYS-OH

Boc-Leu...enk...Lys-OH (100mg; 0.125 mmol) was dissolved in 5 mL of DMF:DCM(1:1) and stirred at 10 °C under nitrogen. To this clear solution TEA (17.5 µL) and a solution of succinimidyl cetyl-PEG₂, dissolved in 1 mL of DCM were added.

5 After 1.5h (TLC showed single product) the solvent was removed under reduced pressure at room temperature and reaction mixture was admixed with water and extracted with ethyl acetate (10 mL x 3). The organic extract was sequentially washed with water, brine, dried and evaporated to a solid.

5.4.1 PURIFICATION OF DERIVATIZED BOC-LEU...ENK...LYS-OH ON SILICA GEL COLUMN

10 The derivatized blocked enkephalin was purified on silica gel column using methanol-chloroform (5% methanol-chloroform) mixture as an eluting solvent. After evaporation of desired fraction 100 mg pure compound was obtained. A product yield of 100 mg was obtained after removal of the solvent.

5.4.2 DEBLOCKING OF BUTYLOXYCARBONYL GROUP FROM DERIVATIZED LEU...ENK

15 Derivatized Boc-Leu...enk. (100 mg; 0.0866 mmol) was treated with 0.4ml of TFA-DCM (1:1) for 30 min. at room temperature. The solvent was removed under reduced pressure. The solid was redissolved in 2ml of methanol, filtered and evaporated; 80 mg of pure product was obtained.

20 5.5 SYNTHESIS OF AMPHIPHILIC OLIGOMER-ENKEPHALIN CONJUGATES

5.5.1 A GENERAL SCHEME FOR SYNTHESIS OF NON-HYDROLYZABLE AND HYDROLYZABLE CONJUGATES

25 One-hundred milligrams of enkephalin (100 mg; 0.142 mmol) was dissolved in dry dimethylformamide (5 mL) at room temperature. P-nitrophenol or N-hydroxysuccinimide activated (carbonate or ester) of amphiphilic oligomer (1.1 mole equivalent) was dissolved in 1 mL tetrahydrofuran and added to above solution and stirred at room

temperature over 1.5 hours. The extent of the reaction was monitored by a reverse phase (C-18) HPLC using isopropanol/water (0.1% trifluoroacetic acid) gradient system. Reaction mixture was evaporated under reduced pressure and the contents were dissolved in an isopropanol-water mixture. This mixture was purified on a 22 mm preparative HPLC column (C-8) with a solvent gradient system made of either isopropanol/water (0.1% trifluoroacetic acid) or acetonitrile/water (0.1% trifluoroacetic acid) to give pure monoconjugated and diconjugated enkephalins. The solvent was evaporated at low temperature (<20 °C) to give dry produce. The purity of the product was analyzed by reverse phase analytical HPLC, and the MW information was obtained by MALDI (TOF)-mass spectral technique.

5.5.2 SYNTHESIS OF CHOLESTEROL-PEG₂ HYDROLYZABLE AMPHIPHILIC OLIGOMER

PEG₂ diacid (3,6,9-trioxaundecanoic diacid, 10 g) was dissolved in dry chloroform (50 mL) and added dropwise to oxalylchloride at room temperature under dry condition in the presence of catalytic amount of dimethylformamide. The reaction was stirred for 6 hours and the solvent and excess of reagent was stripped off to give an oily residue.

Above residue was dissolved in chloroform (50 mL) and to this was added cholesterol (1.05 mole equivalent) in chloroform (50mL) and triethylamine (1 mole equivalent) over 30 minutes at 5 °C. The reaction was stirred at 15 °C over 2 hours. To this was added N-hydroxysuccinimide (1 mole equivalent) in chloroform (50mL) and followed by triethylamine (1 equivalent) at 5 °C and allowed to stir overnight. Solvent was stripped off and the product was extracted with ethylacetate. Crude product was purified on a silica gel column with 1:10 methanol/chloroform solvent system to obtain activated amphiphilic oligomer in 80% yield.

5.6 MOLECULAR WEIGHT INFORMATION OF ENKEPHALIN CONJUGATES OBTAINED BY MALDI (TOF)-MS

Enkephalin Conjugate	Expected M. W.	Observed M. W.
Cholesterol-PEG ₂	1274	1275

DHA- PEG ₂	1144.4	1144.3
Linolenic- PEG ₂	1093.4	1093.3
Cetyl- PEG ₂	Avg. 1059	Avg. 1032
Palmitate- PEG ₃	1116	1115.6
Cetyl- PEG ₃	1101	1101.12

These results demonstrate that the reactions resulted in monoconjugates, i.e., each peptide was coupled to only one oligomer. It is significant to note that a single conjugate is sufficient to impart amphiphilic properties.

5 5.7 STABILITY OF MET ENKEPHALIN-LYS (ENKEPHALIN) AND ITS AMPHIPHILIC OLIGOMER CONJUGATES IN RAT BRAIN HOMOGENATE

Met enkephalin-lys and its conjugates (Cetyl-PEG₂, Cetyl-PEG₃ and Palmitate-PEG₃) were incubated in 2% rat brain homogenate. Samples were drawn over time intervals and the amount of the substance remaining was measured by a HPLC method.

10 Following experimental procedure was used for the study.

Procedure: A 2% rat brain homogenate was prepared by homogenizing freshly perfused (PBS buffer) rat brain in PBS buffer (pH 7.4). Two 3-mL aliquots of the homogenate were equilibrated at 37 °C in a water bath. To one unmodified enkephalin was added and to other modified (conjugate) was added, resulting in a final concentration of 15 60ug/mL of peptide. At time 0, 1, 2, 3, 5, 15, 30, and 60 minutes, 200 uL of aliquot was withdrawn and quenched with 200 uL of the quenching agent (1% trifluoroacetic acid in acetonitrile/isopropanol or 1% trichloroacetic acid in water). The sample solutions were vortexed and centrifuged at 7000RPM. The supernatant was analyzed by a HPLC method using a gradient of 10 to 100% isopropanol/water (0.1% trifluoroacetic acid) on a 20 C-18 column.

Figure 2 shows the stability of the cetyl-PEG₂-enkephalin conjugate as compared to free met-enkephalin-lys. Figure 3 shows the stability of the cetyl-PEG₃-enkephalin as

compared to met-enkephalin-lysine. Figure 4 shows palmitate-PEG₃-enk (hydrolyzable) conjugate as compared to met-enkephalin-enk.

5.7.1 EXTRACTION AND DETECTION OF ENKEPHALIN CONJUGATES FROM THE BRAIN OF DOSED RATS

5 5.7.1.1 PROCEDURE

The following procedure was used to identify the presence of conjugate from the brain specimen of animals dosed with 5 mg/kg cetyl-PEG₂-enkephalin.

After 10 minutes of dosing, the brain of the animal was perfused with 1.5% trifluoroacetic acid in PBS solution, and the brain was removed and frozen at -70 °C.

10 The brain was homogenized with 1mL of 1.5% trifluoroacetic acid in PBS solution and the homogenate was extracted with acetonitrile/isopropanol solution. The extract was treated with saturated sodium chloride solution and frozen at -20 °C for 2 hours. The organic layer was isolated and centrifuged at 4000RPM. The supernatant was evaporated and the resulting residue was reconstituted in acetonitrile/isopropanol/water

15 mixture. The reconstituted solution was analyzed by HPLC using a gradient of 10 to 100% isopropanol/water (0.1% trifluoroacetic acid) on a C-18 column. The presence and the concentration of cetyl-PEG₂-enkephalin conjugate in the extract were measured by comparing the retention time and the peak area of standard solution under the same analytical condition. The results are presented in FIGs 5A to 5D.

20 5.7.1.2 RESULTS

The results demonstrate that monoconjugates were isolated from brain tissue. FIG 5A shows a peak produced by cetyl enkephalin standard, while 5B shows a corresponding peak demonstrating that cetyl enkephalin was actually present in the brain extract. In contrast, neither the vehicle (FIG. 5C) nor the unconjugated enkephalin (FIG. 5D)

25 showed a corresponding peak.

5.8 RAT PAW-HOT PLATE TEST

5.8.1 ANIMALS

Adult, male Sprague-Dawley rats weighing 150-175 g were obtained from Charles River Breeding Laboratories (Raleigh, NC) and used for all animal studies. Rats were housed in hanging wire-bottomed cages in a vivarium equipped with a 12:12 light:dark cycle and humidity was maintained between 45-65% with a room temperature of $72 \pm 2^{\circ}\text{C}$. Rats were provided Purina Rodent Chow and tap water ad libitum.

5.8.2 METHODS

Met-enkephalin-lys and met-enkephalin-lys derivatives were assessed for analgesic activity by rat paw-hot plate assay. Rats were given an injection of naloxone at 0.5 mg/kg (s.c.) then administered a single administration of cetyl-enkephalin by the tail vein 10 minutes later at a dose of 5.0 mg/kg. The results as graphically displayed in FIG. 6 demonstrate that Naloxone, an μ -receptor antagonist prevents competitively inhibits binding of cetyl-PEG₂-enkephalin, thus demonstrating that at least part of the activity of cetyl-PEG₂-enkephalin is attributable to binding at the Opioid μ -receptor.

In a separate study, rats were administered cetyl-enkephalin (5.0 mg/kg, i.v.) or clonidine (0.125 mg/kg, i.v.).

The latency to rat paw withdrawal from the hot plate was measured by a Hot Plate Analgesia Meter (Harvard Apparatus Ltd., Kent, England). The temperature of the hot plate was set and calibrated at 52 °C and rats were removed from the heat stimulus by 36 seconds after placement. Latency trials were terminated when the animal was either licking a hind paw or initiating a jump from the plate. Baseline measurements were collected 1 hour prior to drug administration and at various times post-injection, dependent upon the study conducted. All hot plate testing was terminated by 1 hour after drug dosing.

5.8.3 RESULTS

The results are displayed in the following tables and in the Graph of FIG 6. The results demonstrate that while 20 mg/kg enkephalin alone has 0% analgesic effect as compared

to morphine as a baseline, the enkephalin conjugates of the present invention had strong analgesic effects and one conjugate, DHA-PEG-ENK had 130% of the analgesic effect of morphine. The graph of FIG. 7 shows that CETYL-PEG-ENK produces a response and duration comparable to that of clonidine, an α -adrenergic receptor agonist.

ANALGESIC EFFECT OF ENKEPHALIN CONJUGATES IN RATS				
Drug or Conjugate	Dose (mg/kg)	Number of Rats	Mean Analgesia as Compared with Morphine at 3 mg/Kg*	
			@ 5 min	@ 30 min
Morphine	3	8	100%	100%
Enkephalin	20	7	0%	0%
Cetyl-PEG-ENK	5	8	84%	75%
DHA-PEG-ENK	20	8	130%	67%
Cholesterol-PEG-ENK	5	8	80%	68%
Linolenic-PEG-ENK	10	8	77%	73%

5

5.9 AGONIST-STIMULATED [35 S] GTP γ S BINDING IN BRAIN SECTIONS.

5.9.1 MATERIALS

Male Sprague-Dawley rats (200 g) were purchased from Zivic-Miller (Zelienople, PA).

10 [35 S]GTP γ S (1250 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO), adenosine deaminase, and GDP were obtained from Sigma Chemical Co. (St. Louis, MO). Reflections® autoradiography film was purchased from New England Nuclear Corp. (Boston, MA). All other reagent grade chemicals were obtained from Sigma Chemical Co. or Fisher.

15 5.9.2 AGONIST-STIMULATED [35 S] GTP γ S BINDING IN BRAIN SECTIONS.

Agonist-stimulated [35 S]GTP γ S autoradiography was performed as described by Sim *et al.* *Proc. Nat'l. Acad. Sci. USA* 1992 Pg. 7242 – 7246. Animals were sacrificed by decapitation and brains were removed and frozen in isopentane at -30°C. Coronal and horizontal brain sections were cut on a cryostat maintained at -20°C. Sections were

5 incubated in assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, pH 7.4) at 25°C for 10 min. Sections were then incubated in assay buffer containing 2 mM GDP, protease inhibitor cocktail (10 μ l/ml of a solution containing 0.2 mg/ml each of bestatin, leupeptin, pepstatin A and aprotinin), and adenosine deaminase (9.5 mU/ml) at 25° C for 15 min. Sections were then incubated in assay buffer with GDP, 0.04 nM

10 [35 S]GTP γ S and appropriate agonist at 25°C for 2 hours. The agonists were: 10 μ M DAMGO, 10 μ M cetyl-enkephalin and 10 μ M cetyl-TEG-enkephalin. Basal binding was assessed in the absence of agonist. Slides were rinsed twice for 2 min each in cold Tris buffer (50 mM Tris-HCl, pH 7.4) and once in deionized H₂O. Slides were dried overnight and exposed to film for 72 hours. Films were digitized with a Sony XC-77 video camera

15 and analyzed using the NIH IMAGE program for Macintosh computers.

5.9.3 RESULTS

Results show that cetyl-TEG-enkephalin stimulates of [35 S]GTP γ S binding. The anatomical distribution of the binding is consistent with that of μ Opioid receptors. These results demonstrate that cetyl-TEG-enkephalin does not simply bind the receptor but

20 also activates the receptor, causing the receptor to bind to G-protein. This activation provides further corroborative evidence that cetyl-TEG-enkephalin directly stimulates analgesia.

THE CLAIMS

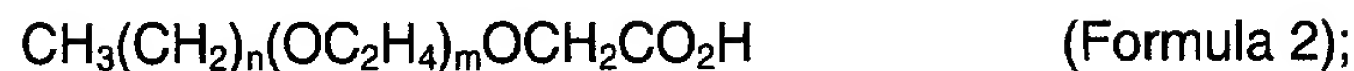
We claim:

1. An amphiphilic drug-oligomer conjugate comprising a therapeutic compound conjugated to an oligomer, wherein the oligomer comprises a lipophilic moiety coupled to a hydrophilic moiety.
2. The amphiphilic drug-oligomer conjugate of claim 1 further characterized in that said conjugate exhibits activity without cleavage of the therapeutic compound from the oligomer.
3. The amphiphilic drug-oligomer conjugate of claim 1 further characterized in that said therapeutic compound does not exhibit activity without cleavage of the therapeutic compound from the oligomer.
4. The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is a peptide or protein.
5. The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is a peptide and the peptide is selected from the group consisting of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminase, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystokinin, clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen activator, trypsin, vasopressin and analogues and active fragments thereof.

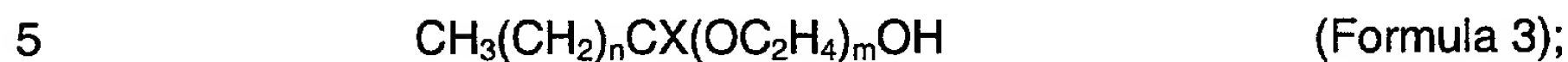
6. The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is an Opioid receptor agonist, antagonist or partial agonist/partial antagonist.
- 5 7. The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is [met⁵]enkephalin.
8. The amphiphilic drug-oligomer conjugate of claim 1 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a hydrolyzable bond.
9. The amphiphilic drug-oligomer conjugate of claim 1 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a non-hydrolyzable bond.
- 10 10. The The amphiphilic drug-oligomer conjugate of claim 1 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a bond selected from the group consisting of: amide bond, carbamate bond, carbonate bond and ester bond.
11. The amphiphilic drug-oligomer conjugate of claim 1 wherein the oligomer moiety is coupled to the drug moiety by a bond selected from the group consisting of
15 amide bond, carbamate bond, carbonate, and ester.
12. The amphiphilic drug-oligomer conjugate of claim 1 wherein the lipophilic moiety is selected from the group consisting of fatty acids, C₁₋₂₆alkyls, cholesterol, and adamantane.
13. The amphiphilic drug-oligomer conjugate of claim 1 wherein the hydrophilic
20 moiety is selected from the group consisting of sugars or PEG₁₋₇.
14. The amphiphilic drug-oligomer conjugate of claim 1 wherein the hydrophilic moiety comprises a sugar and the sugar is selected from the group consisting of: amino sugars and non-amino sugars.
15. The amphiphilic drug-oligomer conjugate of claim 1 wherein the oligomer is
25 selected from the group consisting of:



wherein $n=3$ to 25 and $m=1$ to 6;



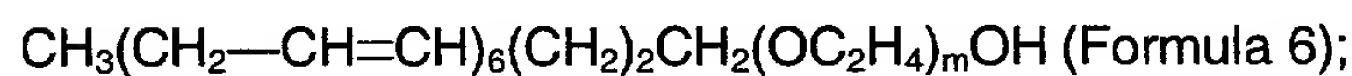
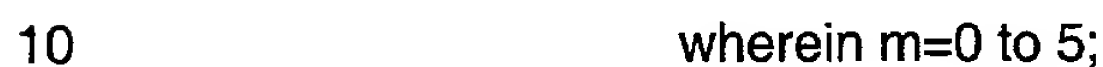
wherein $n=3$ to 25 and $m=1$ to 7;



wherein $n=3$ to 25, $m=1$ to 7 and $X=\text{O}$ or N ;



wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



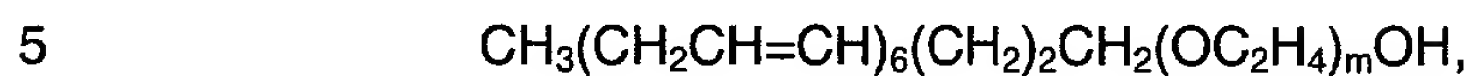
wherein $m=0$ to 7;



wherein $m=1$ to 7 and $X=\text{N}$ or O .

- 15 16. The method of claim 1 wherein the hydrophilic moiety comprises a sugar and the sugar is selected from the group consisting of amino sugars and non-amino sugars.

17. The method of claim 1 wherein the hydrophilic moiety comprises a monosaccharide.
18. An amphiphilic drug-oligomer conjugate of claim 1 wherein the oligomer is selected from the group consisting of:



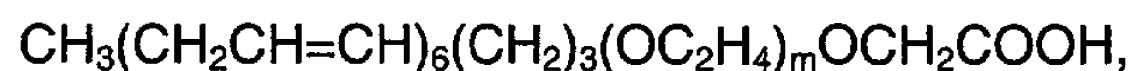
where $m=1$ to 7;



where $m=1$ to 7;



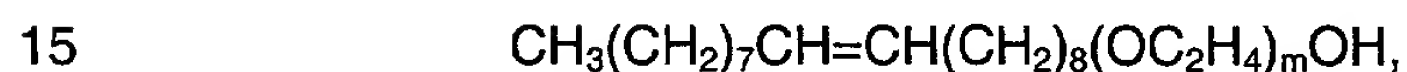
10 where $m=1$ to 6;



where $m=1$ to 6;



where $m=1$ to 6;



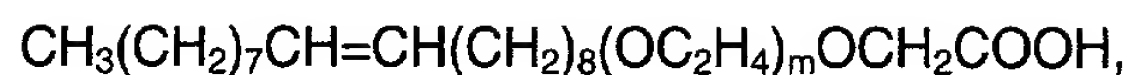
where $m=1$ to 7;



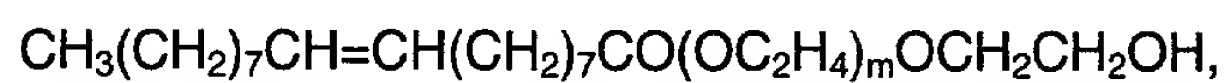
where $m=1$ to 7;



where m=1 to 6;

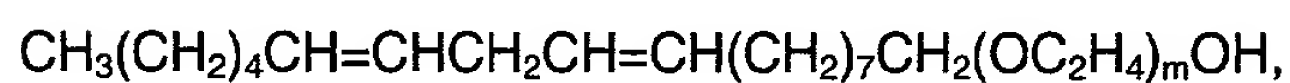


where m=1 to 6;

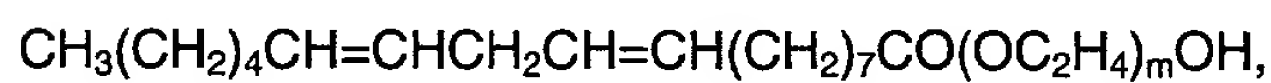


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where m=1 to 6;

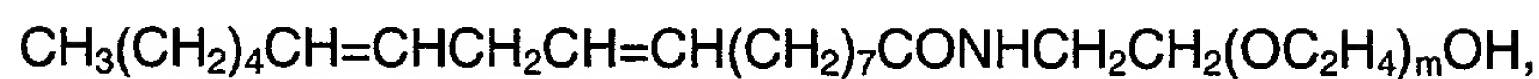


where m=1 to 6;

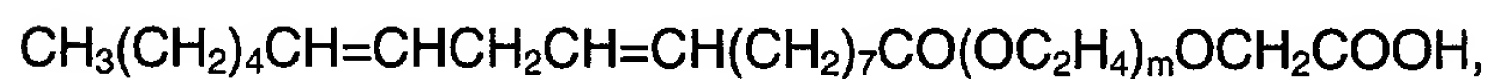


where m=1 to 7;

10



where m=1 to 6;



where m=1 to 6;



15

where m=1 to 6;



where m=1 to 7;



where m=1 to 7;

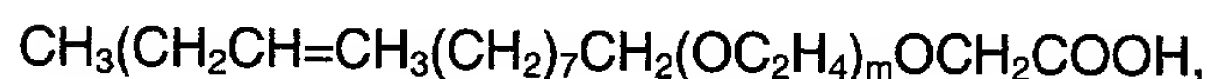


where m=1 to 6;



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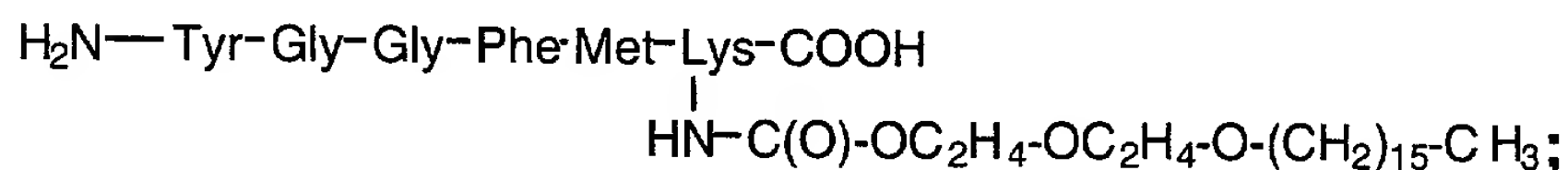
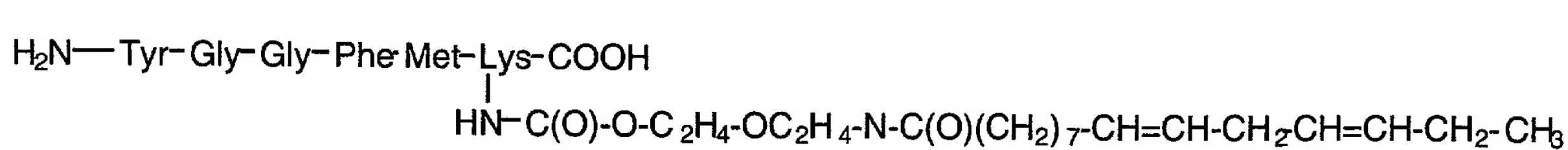
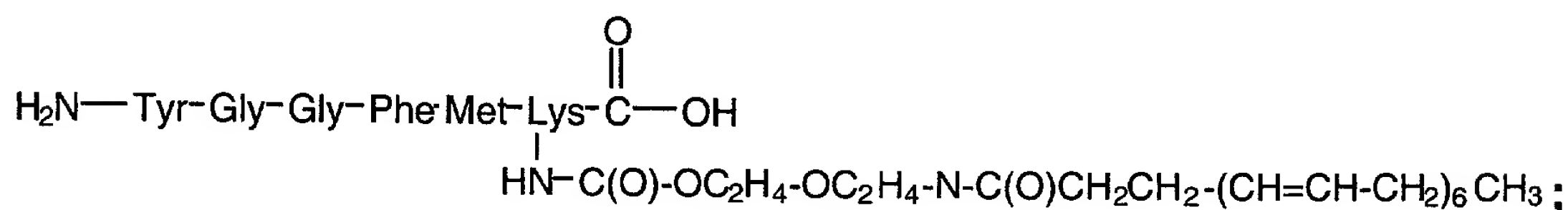
where m=1 to 6; and



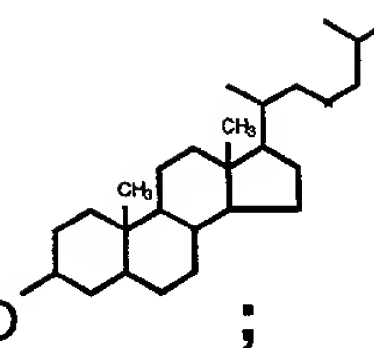
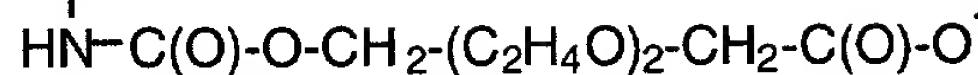
where m=1 to 6.

19. The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is a peptide having an added N-terminal residue selected from the group consisting of proline or alanine.

20. An amphiphilic oligomer-enkephalin conjugate selected from the group consisting of:

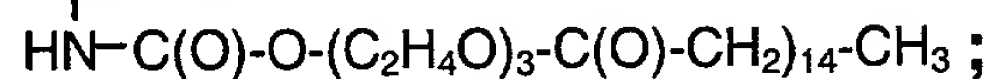


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;

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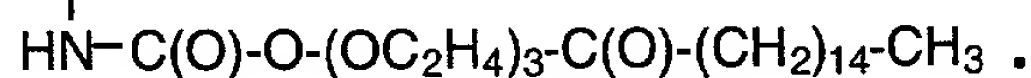
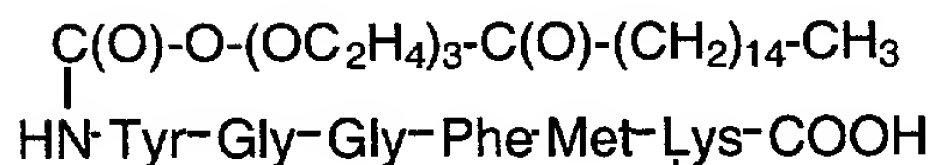


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20

; and

25



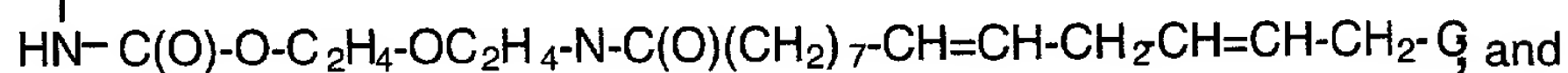
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21. An amphiphilic oligomer-enkephalin conjugate wherein the oligomer is comprised of a lipophile and a hydrophile and the lipophile is coupled to the hydrophile by a hydrolyzable bond, said conjugate being selected from the group consisting of:

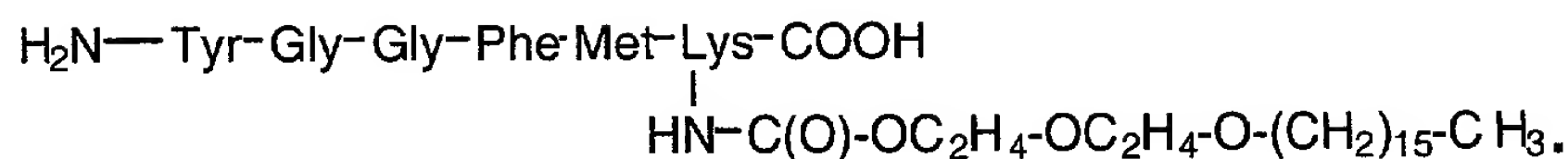
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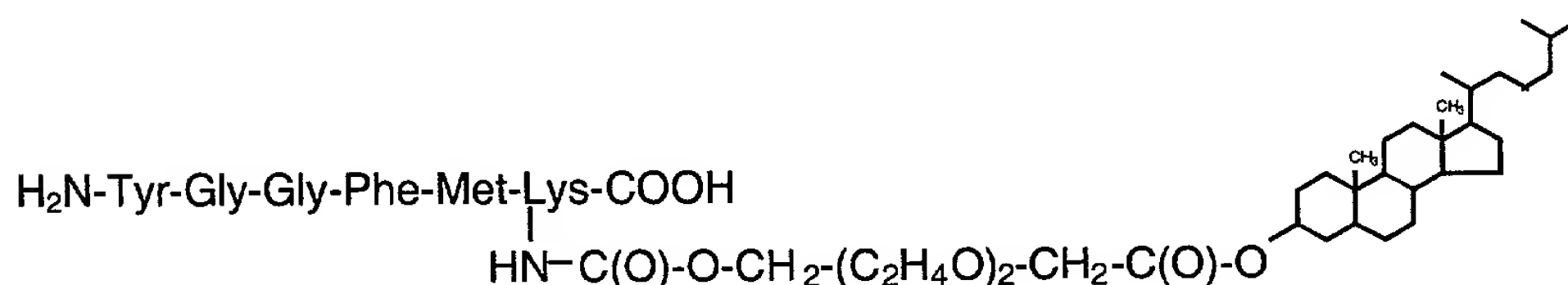
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22. An amphiphilic oligomer-enkephalin conjugate wherein the oligomer is comprised of a lipophile and a hydrophile and the lipophile is coupled to the hydrophile by a non-hydrolyzable bond, said conjugate being selected from the group consisting of:

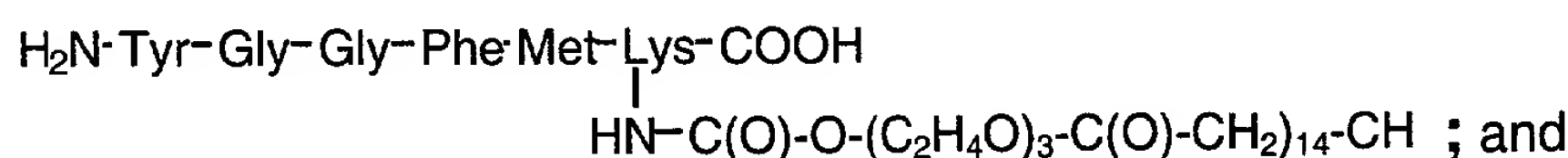
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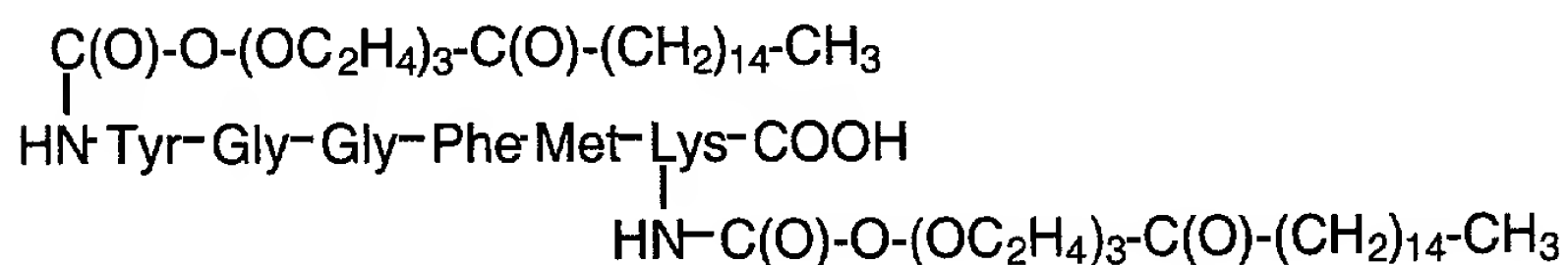
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23. A method for activating a receptor comprising bringing said receptor into contact with an amphiphilic drug-oligomer conjugate comprising a therapeutic compound conjugated to an oligomer, wherein the oligomer comprises a lipophilic moiety coupled with to a hydrophilic moiety.

40

24. The method of claim 18 further characterized in that said conjugate exhibits activity in the without cleavage of the therapeutic compound from the oligomer.
25. The method of claim 18 wherein the receptor is a G-protein coupled receptor.
26. The method of claim 18 wherein the receptor is an Opioid receptor.
- 5 27. The method of claim 18 wherein the receptor is a Opioid receptor; selected from the group consisting of δ , μ , and κ .
28. The method of claim 18 wherein the hydrophilic moiety is selected from the group consisting of sugar and PEG₁₋₇.
- 10 29. The method of claim 18 wherein the hydrophilic moiety is selected from the group consisting of fatty acid, alkyl 1-26, cholesterol and adamantane.
30. The method of claim 18 wherein the therapeutic compound is a peptide having an added N-terminal residue selected from the group consisting of proline, and alanine.
- 15 31. The method of claim 18 wherein the therapeutic compound is a peptide or protein.
32. The method of claim 18 wherein the therapeutic compound is a peptide and the peptide is selected from the group consisting of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminease, asparaginase, caerulein, 20 calcitonin, chemotrypsin, cholecystokinin, clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, 25 soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen

activator, trypsin, vasopressin, and analogues and active fragments of such peptides.

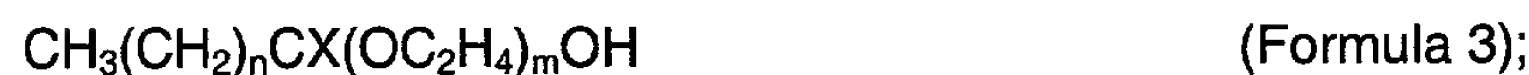
33. The method of claim 18 wherein the amphiphilic oligomer is selected from the group consisting of:



wherein $n=3$ to 25 and $m=1$ to 6;



wherein $n=3$ to 25 and $m=1$ to 7;



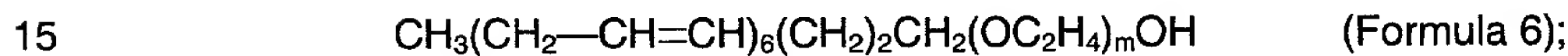
10 wherein $n=\underline{3}$ to 25 , $m=1$ to 7 and $X=\text{O}$ or N ;



wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 5;



wherein $m=0$ to 7;



wherein $m=1$ to 7 and $X=\text{N}$ or O .

34. The method of claim 18 wherein the hydrophilic moiety is coupled to the hydrophobic moiety by a hydrolyzable bond.
35. The method of claim 18 wherein the hydrophilic moiety is coupled to the hydrophobic moiety by a non-hydrolyzable bond
- 5 36. A method for delivering a therapeutic compound across the blood-brain barrier comprising administering to a subject an amphiphilic drug-oligomer conjugate comprising a therapeutic compound conjugated to an oligomer, wherein the oligomer comprises a lipophilic moiety coupled to a hydrophilic moiety.
- 10 37. The method of claim 31 wherein the therapeutic compound is a peptide or protein.
- 15 38. The method of claim 31 wherein the therapeutic compound is a peptide and the peptide is selected from the group consisting of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine deaminase, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystokinin, clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins, erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin, non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin, superoxide dismutase, thyroid stimulating hormone, tissue plasminogen activator, trypsin, vasopressin, and analogues and active fragments of such peptides.
- 20 39. The method of claim 31 wherein the therapeutic compound is [met⁵]enkephalin.
- 25 40. The method of claim 31 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a hydrolyzable bond.

41. The method of claim 31 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a non-hydrolyzable bond.
42. The method of claim 31 wherein the lipophilic moiety is coupled to the hydrophilic moiety by a bond selected from the group consisting of: amide bond, carbamate bond, carbonate bond and ester bond.
43. The method of claim 31 wherein the oligomer moiety is coupled to the drug moiety by a bond selected from the group consisting of amide bond, carbonate bond, carbamate bond, and ester bond.
44. The method of claim 31 wherein the lipophilic moiety is selected from the group consisting of fatty acids, C₁₋₂₆alkyls, and cholesterol.
45. The method of claim 31 wherein the hydrophilic moiety is selected from the group consisting of sugars, and PEG₁₋₇.
46. A method for inducing analgesia in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of an amphiphilic drug-oligomer conjugate comprising enkephalin conjugated to an oligomer, wherein the oligomer comprises a lipophilic moiety coupled to a hydrophilic moiety.
47. The method of claim 41 wherein the therapeutic compound is [met⁵]enkephalin.
48. The method of claim 41 wherein the lipophilic moiety is selected from the group consisting of fatty acids, C₁₋₂₆ alkyls, and cholesterol.
49. The method of claim 41 wherein the one or more hydrophilic moieties are selected from the group consisting of sugars and PEG.
50. The method of claim 41 wherein the hydrophilic moiety comprises a sugar and the sugar is selected from the group consisting of amino sugars and non-amino sugars.

51. The method of claim 41 wherein the oligomer is selected from the group consisting of:



wherein $n=3$ to 25 and $m=1$ to 6;



wherein $n=3$ to 25 and $m=1$ to 7;



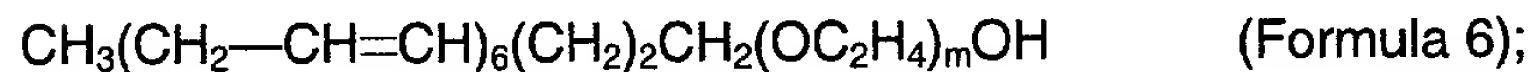
wherein $n=3$ to 25, $m=1$ to 7 and $X=\text{O}$ or N ;



10 wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 4 and $\text{R}=\text{cholesterol}$ or adamantane ;



wherein $m=0$ to 7;



wherein $m=1$ to 7 and $X=\text{N}$ or O .

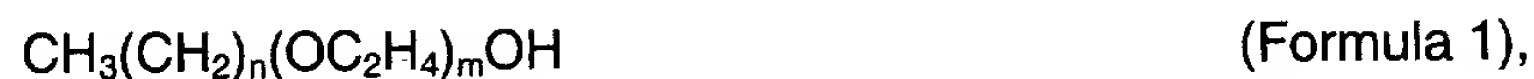
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53. A method for altering the binding affinity of a peptide to its receptor comprising
 5 conjugating the peptide to an amphiphilic oligomer comprising a lipophilic moiety
 coupled to a hydrophilic moiety.
54. The method according to claim 48 further characterized in that the binding affinity
 is increased.
55. The method according to claim 48 further characterized in that the binding affinity
 10 is reduced.
56. The method of claim 48 wherein the peptide is a peptide or protein.
57. The method of claim 48 wherein the peptide is selected from the group consisting
 of: enkephalin, adrenocorticotrophic hormone, adenosine deaminase
 15 ribonuclease, alkaline phosphatase, angiotensin, antibodies, arginase, arginine
 deaminase, asparaginase, caerulein, calcitonin, chemotrypsin, cholecystokinin,
 clotting factors, dynorphins, endorphins, endorphins, enkephalins, enkephalins,
 erythropoietin, gastrin-releasing peptide, glucagon, hemoglobin, hypothalamic
 releasing factors, interferon, katacalcin, motilin, neuropeptide Y, neurotensin,
 20 non-naturally occurring opioids, oxytosin, papain, parathyroid hormone, peptides
 prolactin, soluble CD-4, somatomedin, somatostatin, somatostatin, somatotropin,
 superoxide dismutase, thyroid stimulating hormone, tissue plasminogen
 activator, trypsin, vasopressin, and analogues and fragments of such peptides.
58. The method of claim 48 wherein the peptides is [met⁵]enkephalin.
59. The method of claim 48 wherein the lipophilic moiety is selected from the group
 25 consisting of fatty acids, C₁₋₂₆alkyls, and cholesterol.
60. The method of claim 48 wherein the hydrophilic moiety is selected from the group
 consisting of sugars or PEG₁₋₇.

61. The method of claim 48 wherein the oligomer is selected from the group consisting of:



wherein $n=3$ to 25 and $m=1$ to 6;



wherein $n=3$ to 25 and $m=1$ to 7;



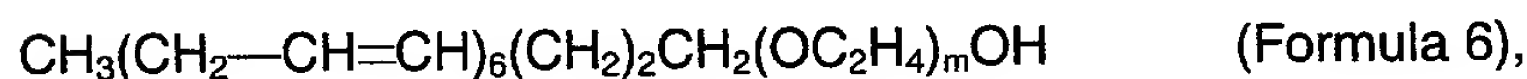
wherein $n=3$ to 25, $m=1$ to 7 and $X=\text{O}$ or N ;



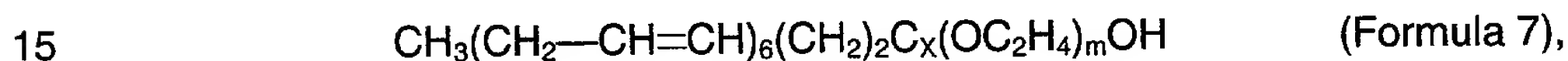
10 wherein $m=0$ to 5 and $\text{R}=\text{cholesterol}$ or adamantane ; or



wherein $m=0$ to 5;



wherein $m=0$ to 7;



wherein $m=1$ to 7 and $X=\text{N}$ or O .

62. The amphiphilic drug-oligomer conjugate of claim 1 wherein the therapeutic compound is a peptide and the peptide is selected from the group consisting of:

Ac-Phe-Arg-Trp-Trp-Tyr-Lys—NH₂ ;

5

Ac-Arg-Trp-Ile-Gly-Trp- Lys —NH₂ ;

Trp-Trp-Pro-Lys-His-Xaa—NH₂ ,

wherein Xaa is a naturally-occurring amino acid;

Trp-Trp-Pro-Xaa—NH₂ ,

wherein Xaa is Lys or Arg;

10

Tyr-Pro-Phe-Gly-Phe-Xaa—NH₂ ,

wherein Xaa is a naturally-occurring amino acid;

(D)Ile-(D)Met-(D)Ser-(D)Trp-(D)Trp-Gly_n-Xaa—NH₂ ,

wherein n is 0 or 1 and wherein Xaa is Gly or the D-form-of a naturally-occurring amino acid;

15

(D)Ile-(D)Met-(D)Thr-(D)Trp-Gly-Xaa—NH₂ ,

wherein Xaa is Gly or the D-form of a naturally-occurring amino acid;

Tyr-A1-B2-C3—NH₂ ,

wherein A1 is (D)Nve or (D)Nle,

B2 is Gly, Phe, or Trp, and

C3 is Trp or Nap;

Pm and red $\{\text{Me}_x\text{H}_y\text{-Tyr-(NMe)}_z\text{-Tyr-Xaa}_z\text{—NH}_2\}$,

x is 0, 1, or 2,

5 y is 0, 1, or 2, and

z is 0 or 1, and

wherein Xaa is Phe, (D)Phe, or NHBzl, with the proviso that x and y together is never greater than 2;

Trp-Trp-Pro-D4-His_z-Xaa_z—NH₂;

10 wherein z is 0 or 1,

wherein D4 is Lys or Arg, and

wherein Xaa is a naturally-occurring amino acid.

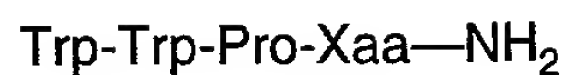
63. The method of claim 18 wherein the therapeutic compound is a peptide and the peptide is selected from the group consisting of:

15 Ac-Phe-Arg-Trp-Trp-Tyr-Lys—NH₂;

Ac-Arg-Trp-Ile-Gly-Trp- Lys —NH₂

Trp-Trp-Pro-Lys-His-Xaa—NH₂

wherein Xaa is a naturally-occurring amino acid;



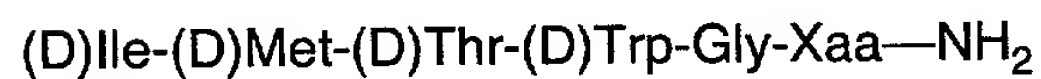
wherein Xaa is Lys or Arg;



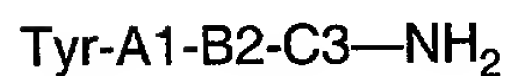
wherein Xaa is a naturally-occurring amino acid;



wherein n is 0 or 1 and wherein Xaa is Gly or the D-form-of a naturally-occurring amino acid;



wherein Xaa is Gly or the D-form of a naturally-occurring amino acid;



wherein A1 is (D)Nve or (D)Nle,

B2 is Gly, Phe, or Trp, and

C3 is Trp or Nap;



x is 0, 1, or 2,

y is 0, 1, or 2, and

z is 0 or 1, and

wherein Xaa is Phe, (D)Phe, or NHBzl, with the proviso that x and y together is never greater than 2;

Trp-Trp-Pro-D4-His_z-Xaa_z—NH₂,

wherein z is 0 or 1,

5

wherein D4 is Lys or Arg, and

wherein Xaa is a naturally-occurring amino acid.

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ABSTRACT

The present invention relates to amphiphilic drug-oligomer conjugates capable of traversing the blood-brain barrier ("BBB") and to methods of making and using such
5 conjugates. An amphiphilic drug-oligomer conjugates comprise a therapeutic compound conjugated to an oligomer, wherein the oligomer comprises a lipophilic moiety coupled to a hydrophilic moiety. The conjugates of the invention further comprise therapeutic agents such as proteins, peptides, nucleosides, nucleotides, antiviral agents, antineoplastic agents, antibiotics, etc., and prodrugs, precursors, derivatives and
10 intermediates thereof, chemically coupled to amphiphilic oligomers.

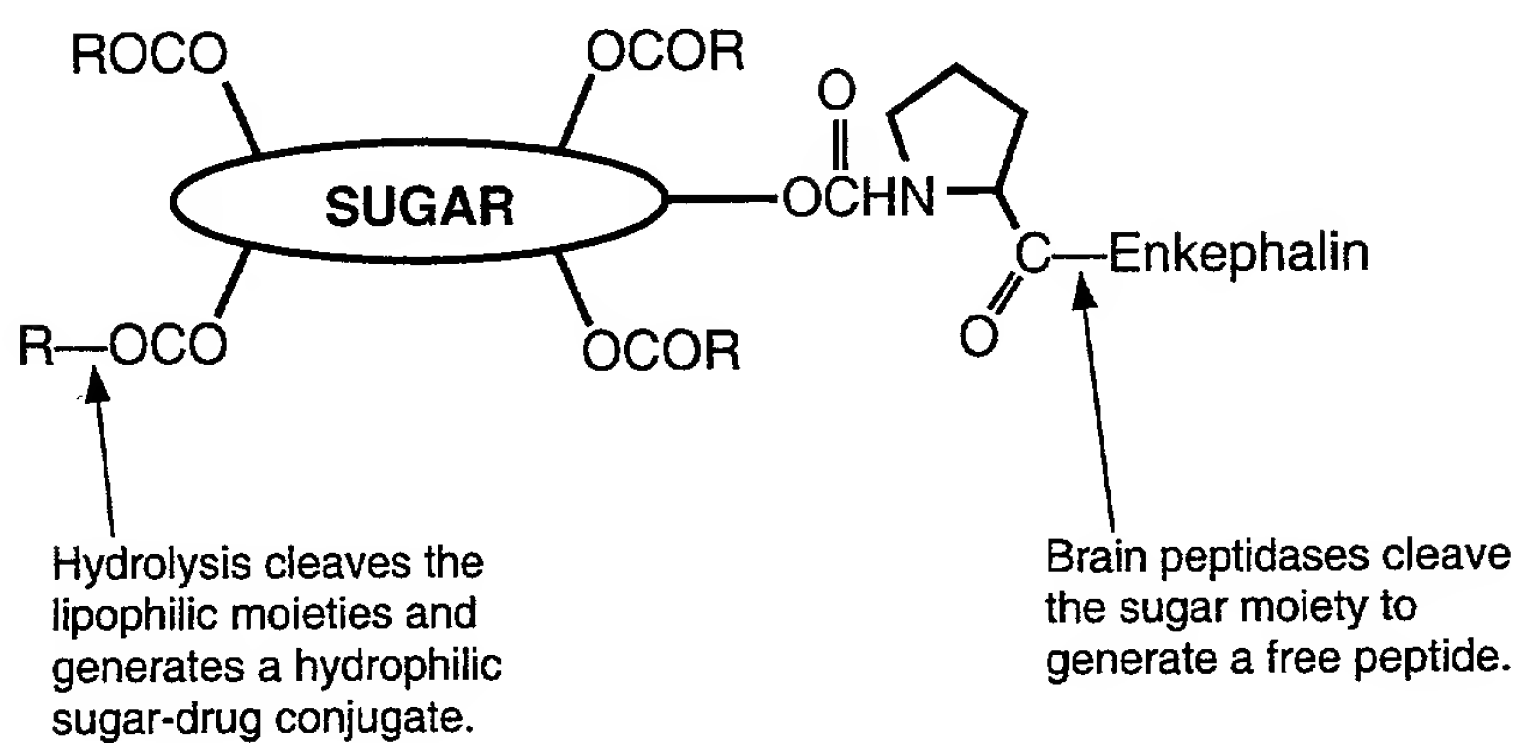
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FIGURE 1A

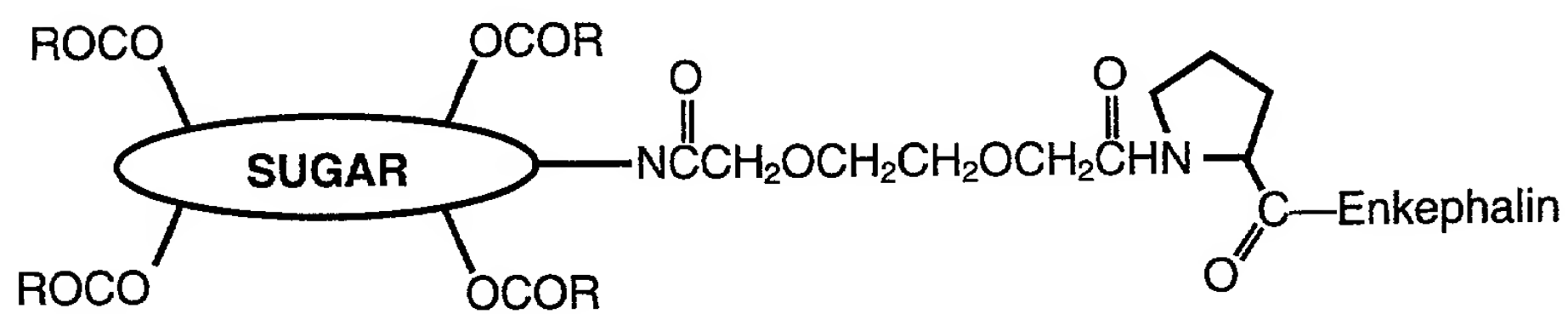


FIGURE 1B

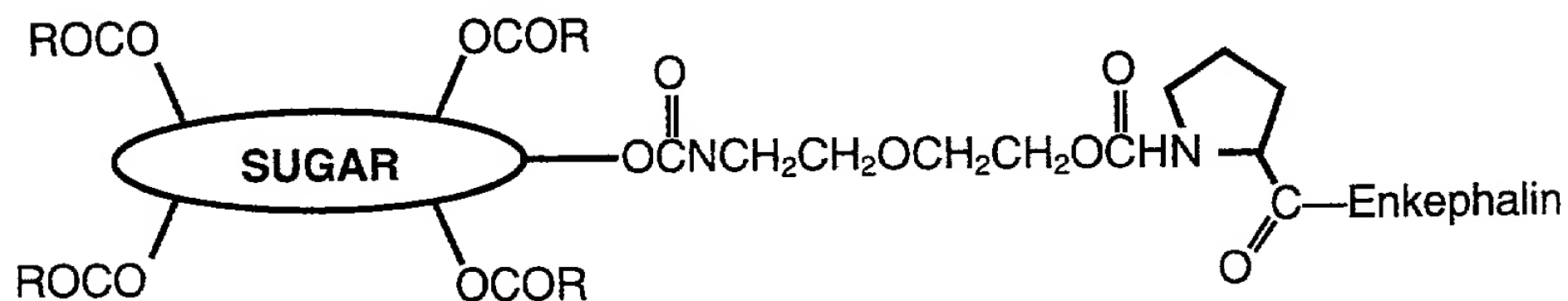


FIGURE 1C

Stability of Enkephalin and Cetyl-PEG₂-Enkephalin in Rat Brain Homogenate

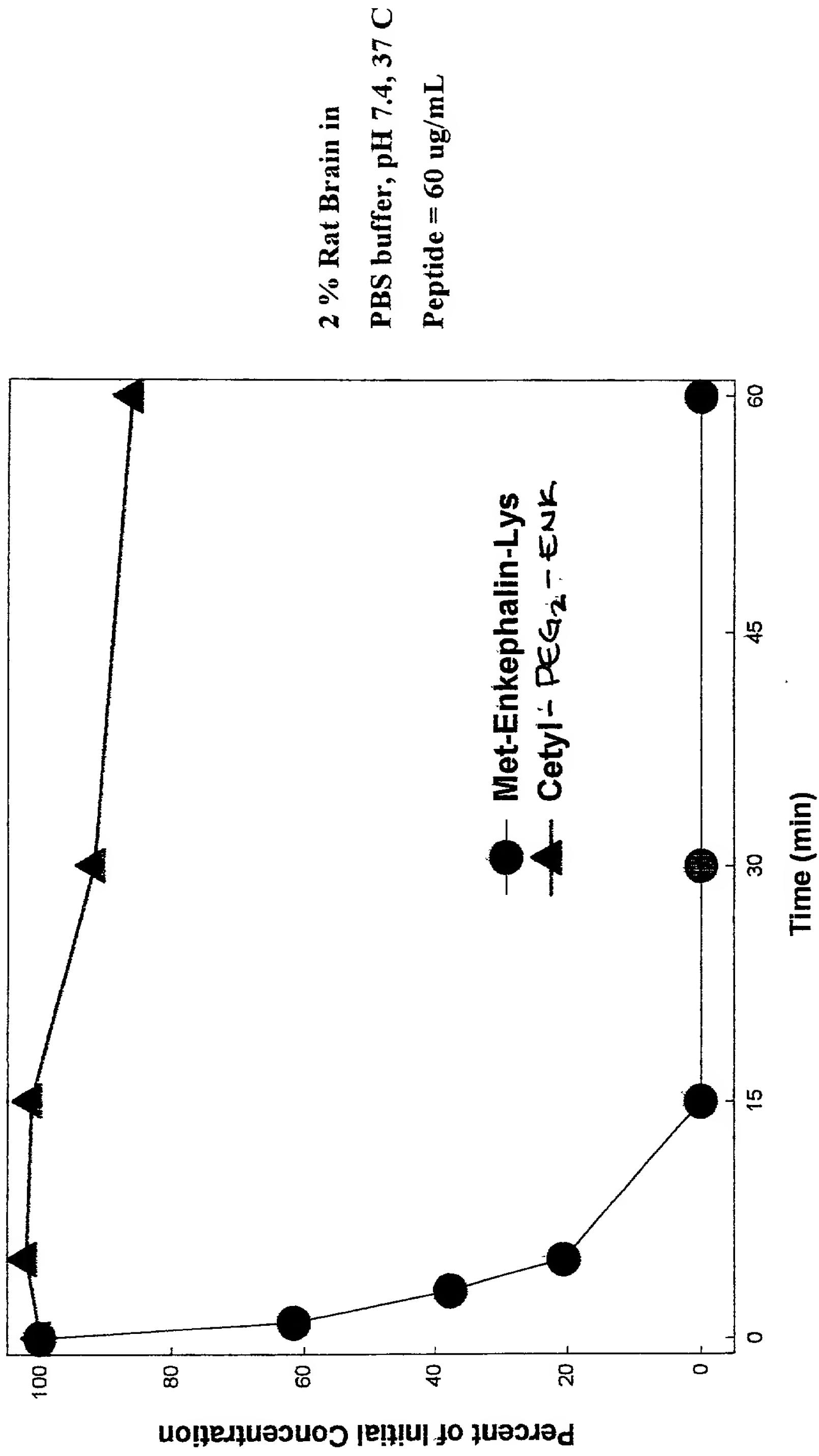


FIGURE 2

Stability of Cetyl-PEG₂ Enkephalin in Rat Brain Homogenate

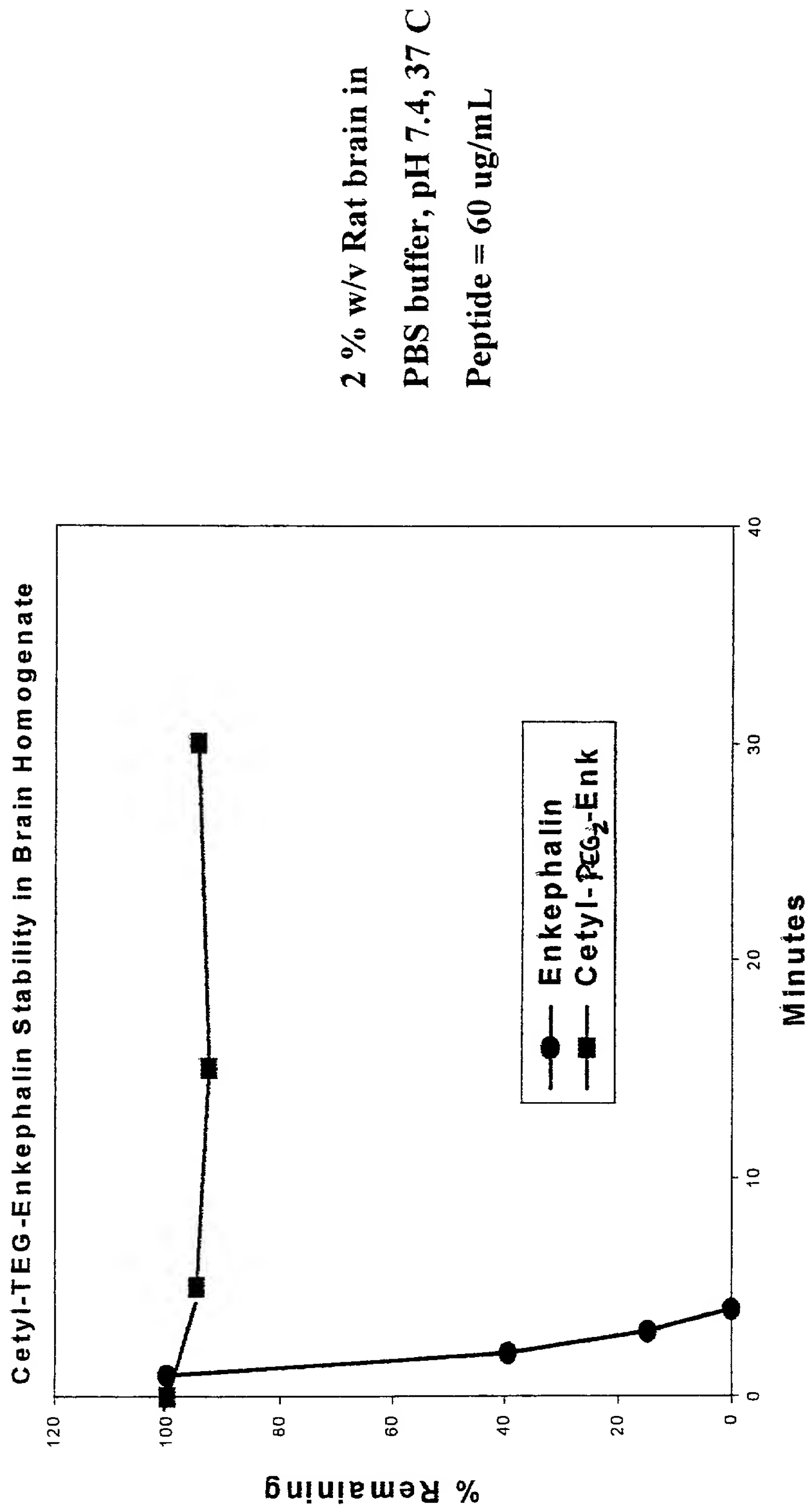


Figure 3

Stability of Palmitate-PEG₃-Enkephalin in Rat Brain Homogenate

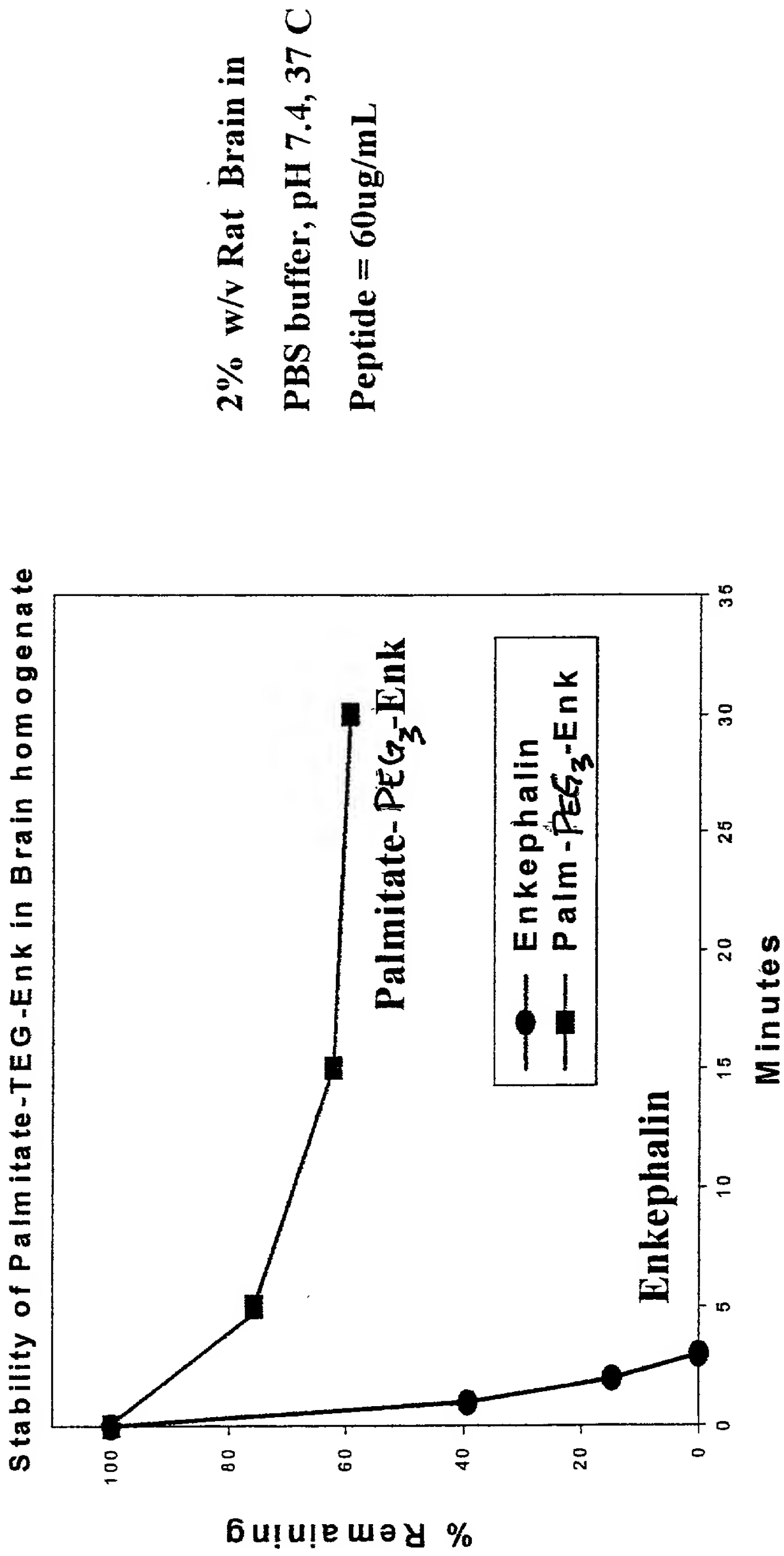


FIGURE 4

Isolation of Cetyl-PEG₂-Enkephalin from the Brain

FIGURE 5A

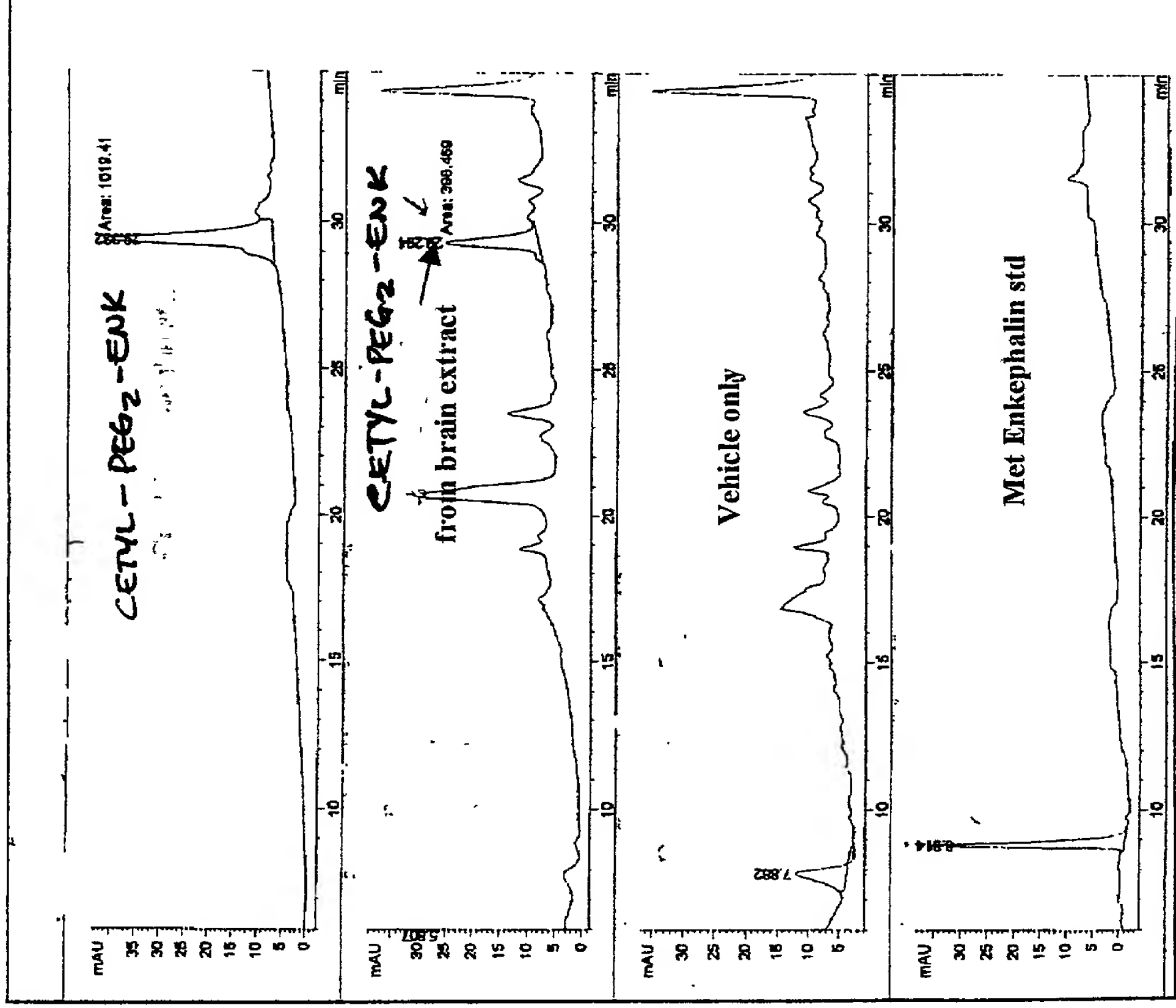


FIGURE 5B

FIGURE 5C

FIGURE 5D

HPLC conditions:
 Column: C-18
 Solvent: solvent A: IPA
 solvent B: Water
 +0.1% TFA
 Gradient: linear

Naloxone Antagonism of Cetyl-PEG₂-Enkephalin-Induced Analgesia

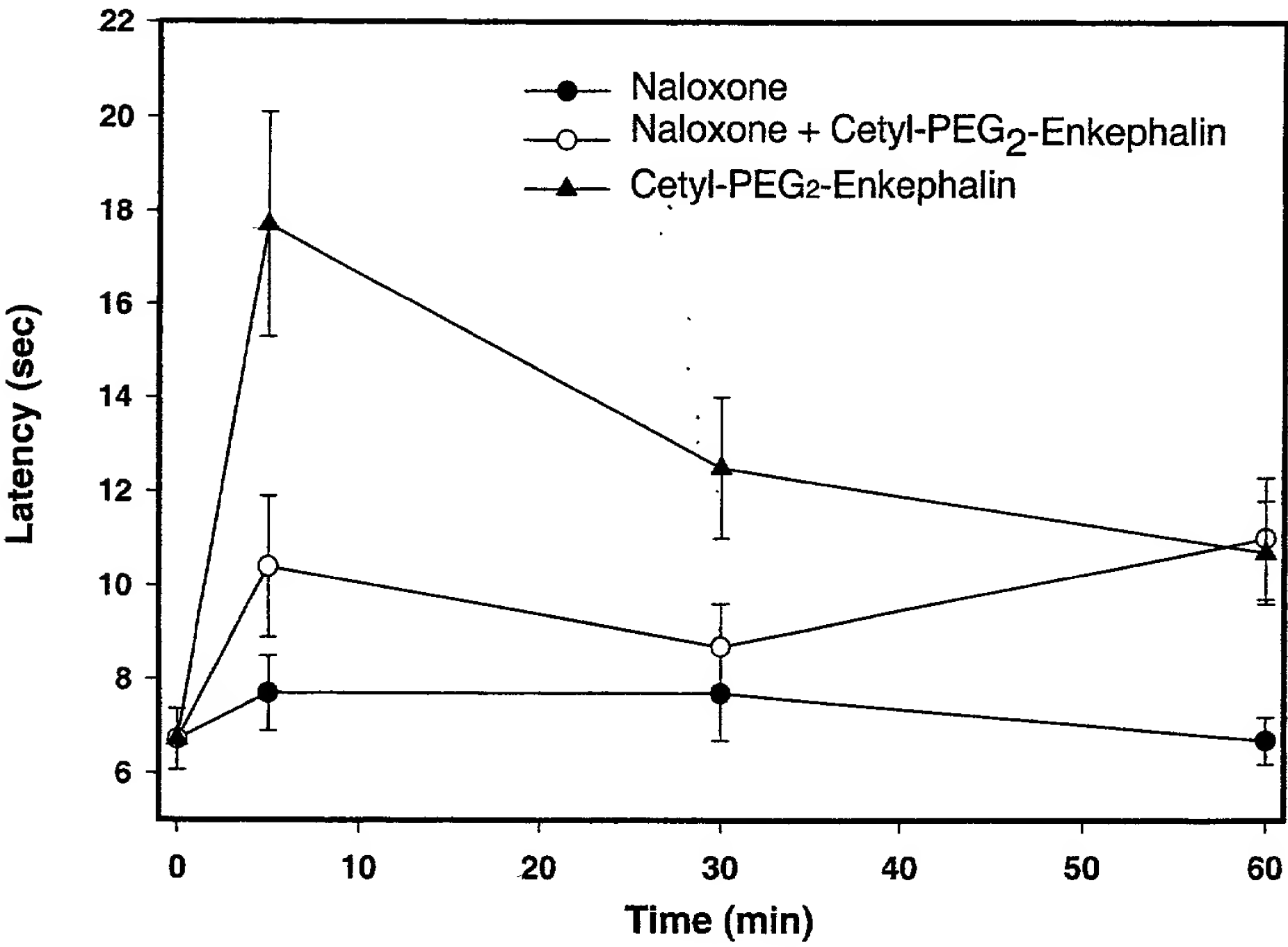


FIGURE 6

Analgesic Effect of a 5 mg/kg IV Dose of Cetyl-PEG₂-Enkephalin Monoconjugate in the Rat Hot-Plate Assay

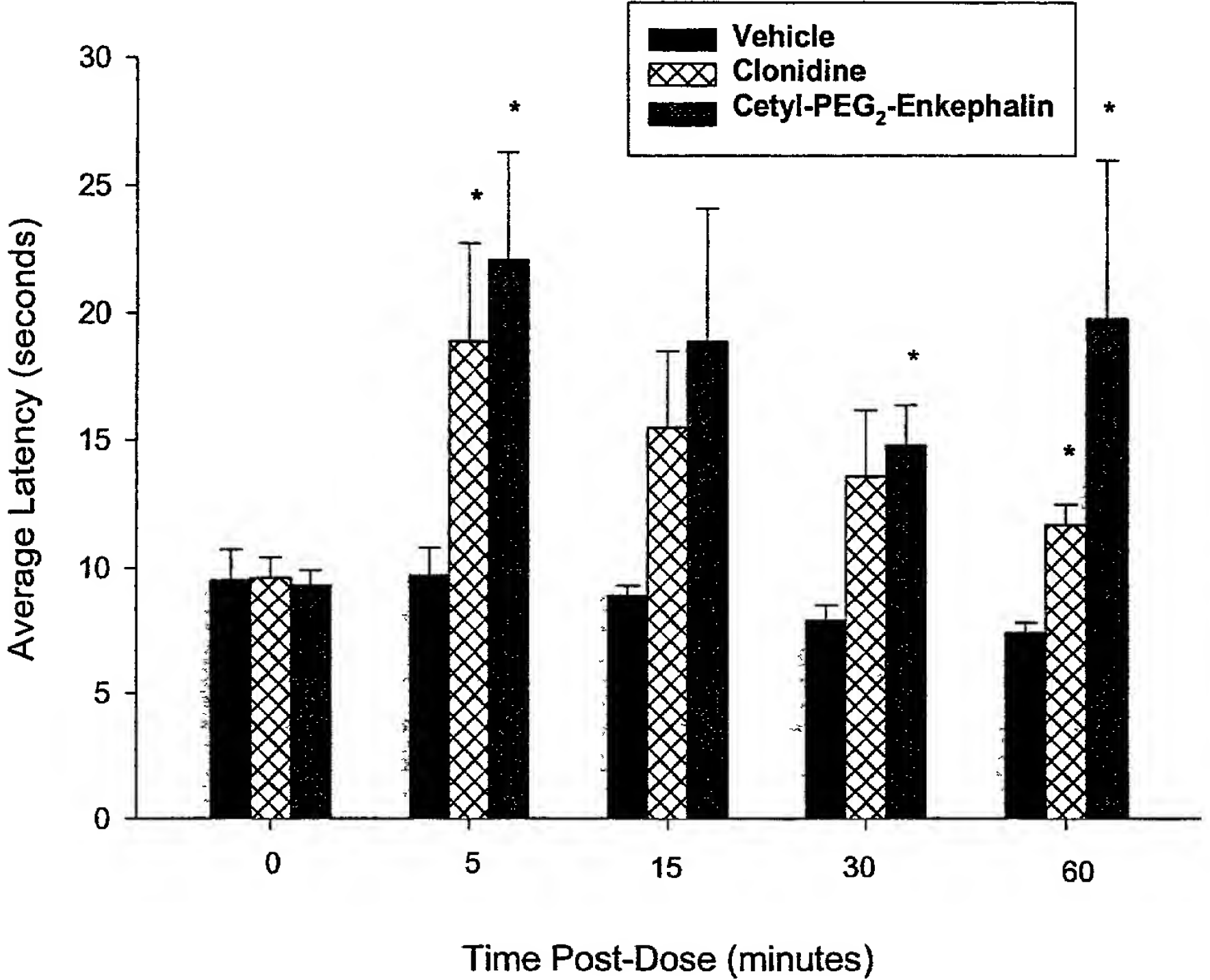


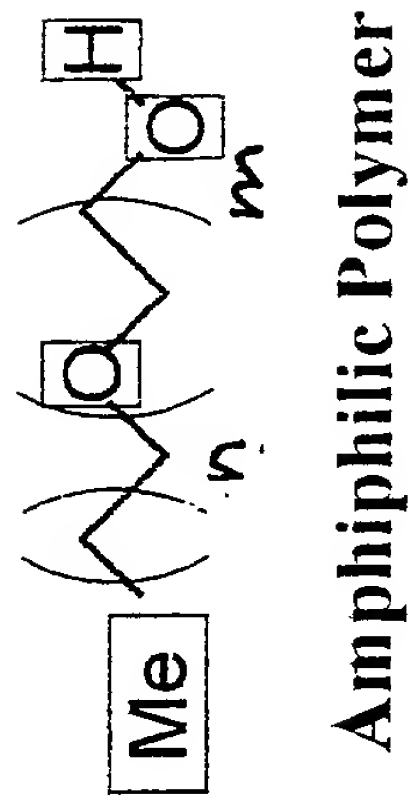
FIGURE 7

COMPARISON OF μ -RECEPTOR BINDING AFFINITY OF ENKEPHALIN CONJUGATES		
DRUG OR CONJUGATE	DETAILED STRUCTURE	% SPECIFIC BINDING*
Naloxone	Naloxone	100
Enkephalin	Met-Enkephalin	67
Cetyl-ENK	Cetyl-PEG ₂ -ENK	100
Chol-ENK	Cholesterol-PEG ₃ -ENK	95
DHA-ENK	DHA-PEG ₂ -ENK	63
Palm-ENK	Palmitate-PEG ₃ -ENK	76
Cetyl-TEG-ENK	Cetyl-PEG ₃ -ENK	100

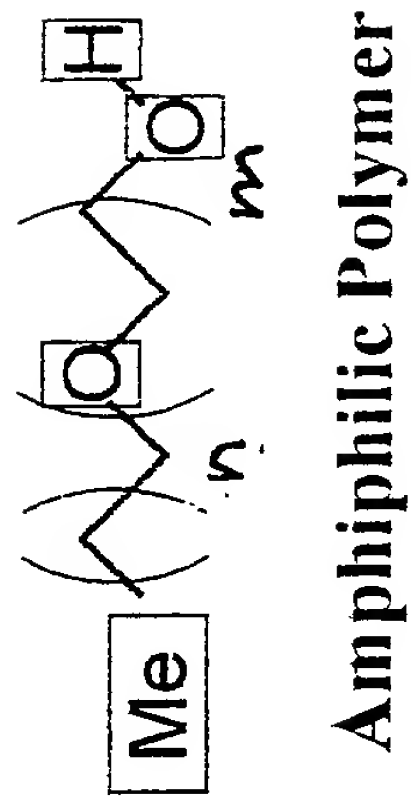
*Data are based on percent inhibition at a concentration of 100nM. The radioligand was DAMGO ([D-Ala², N-Me-Phe-Gly⁵-ol]enkephalin) and naloxone served as the reference.

FIGURE 8

Synthesis of Oligomer



Base





“Free End”



FIGURE 9

Attachment of Oligomer to Enkephalin

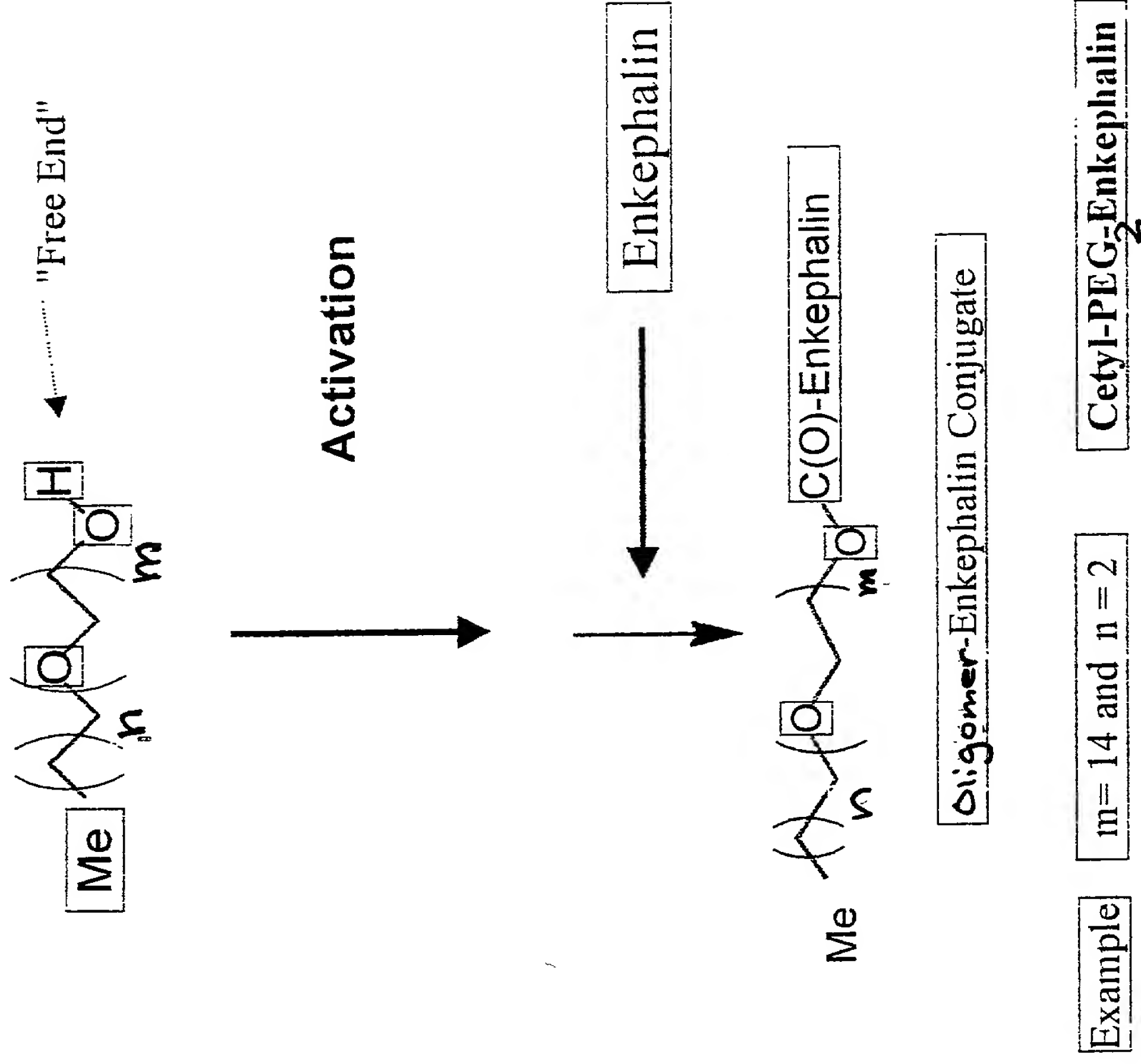


FIGURE 10

A012-113

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name.

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "**Blood-Brain Barrier Therapeutics**", the specification of which

(check one)

☐

is attached hereto.

☒

was filed on August 14, 1998 as Application Serial No. 09/134,803

☐

and was amended _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Claimed

☐ yes ☐ No

(Number)

(Country)

(Day/Month/Year Filed)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this specification is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Number)

(Filing Date)

(Status-Patented, Pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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4012-113

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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